IDENTIFICATION AND CHARACTERISATION OF BAX CHAPERONE PROTEINS WITHIN A NEURONAL CELL MODEL OF INTRINSIC APOPTOTIC CELL DEATH

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DECLARATION

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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ABSTRACT

The pro-apoptotic Bcl-2 family protein, Bax, is an essential component of neuronal apoptotic cell death pathways. Central to the pro-apoptotic function of Bax is its translocation from the cytosol to the outer mitochondrial membrane. Bax is thought to engage in protein-protein interactions with regulatory factors that influence its apoptogenic activity. However the identities of these regulatory proteins have yet to be fully elucidated. This thesis aimed to identify Bax-binding proteins using a proteomics-based approach in an *in vitro* neuronal cell model of the intrinsic apoptotic pathway and to characterise their involvement in Bax-mediated apoptosis using molecular techniques. An *in vitro* neuronal cell model was established using human neuroblastoma SH-SY5Y cells treated with the intrinsic apoptotic pathway inducer, staurosporine. Biochemical hallmarks of the intrinsic pathway, such as Bax translocation and mitochondrial cytochrome c release, together with more general indicators of apoptosis, such as analysis of cellular morphology, caspase-3 activation and internucleosomal DNA fragmentation, were characterised. Endogenous Bax was immunoprecipitated from SH-SY5Y whole cell lysates and bound proteins, separated by 2D gel electrophoresis, were identified by peptide mass fingerprinting to be the heat-shock proteins, Hsc71, GRP78 and Hsp60, and the cytoskeletal proteins, β-actin, tubulin and vimentin. Interactions between endogenous Bax and Hsp60, β-actin and vimentin were confirmed by co-immunoprecipitation. Affinity chromatography with a wild type 21 amino acid C-terminal Bax peptide and a mutant peptide containing a serine to valine substitution at position 184 (S184V), which mimics the membrane-targeting capacity of activated Bax, also identified Hsp60, β-actin and the molecular chaperone nucleophosmin, with the latter binding exclusively to the mutant peptide. These interactions were subsequently confirmed by co-immunoprecipitation. Together, the data suggest that Bax is retained in the cytosol of quiescent cells through association with heat shock and cytoskeletal proteins, and that exposure of the C-terminal domain and subsequent translocation of Bax during apoptosis may be regulated by Hsp60, β-actin and nucleophosmin. The role of nucleophosmin in regulating the translocation of Bax was investigated further. Interaction studies between recombinant Bax and nucleophosmin proteins were

performed using a cell-free amplified luminescent proximity homogeneous assay and demonstrated that nucleophosmin only bound to activated Bax following a detergent-induced conformational alteration. Furthermore, confocal microscopy revealed that nucleophosmin underwent translocation from the nucleolus to the cytosol during a 5h time course of staurosporine treatment and preceded the translocation of Bax to mitochondria. Applying RNA interference against endogenous nucleophosmin mRNA within SH-SY5Y cells attenuated mitochondrial cytochrome c release and procaspase-3 cleavage during staurosporine-induced apoptosis. Together these data demonstrate that nucleophosmin is a novel regulator of Bax bioactivity and represents a novel target for therapeutic intervention in diseases associated with dysfunctional apoptosis.

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PUBLICATIONS ARISING FROM THESIS

PAPERS

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Kerr LE, Birse-Archbold J-LA, Short DM, McGregor AL, Heron I, **MacDonald DC**, Thompson J, Carlson GJ, Kelly JS, McCulloch J and Sharkey J. Nucleophosmin is a novel Bax chaperone that regulates apoptotic cell death. Oncogene (2007). **26**: 2554 – 2562.

Thompson J, Finlayson K, Salvo-Chirnside E, **MacDonald DC**, McCulloch J, Kerr LE and Sharkey J. Characterisation of the Bax-nucleophosmin interaction: the importance of the Bax C-terminus. Manuscript submitted to journal.

ABSTRACTS & POSTERS

MacDonald DC, Birse-Archbold J-LA, Sharkey J and Kerr LE (2006). Nucleophosmin regulates Bax-mediated apoptosis. Abstract P-140. 14th Euroconference on Apoptosis, Sardinia, Italy.

MacDonald DC, Short DM, McCulloch J, Sharkey J and Kerr LE (2005). Proteomic Analysis of Proteins Regulating the Translocation of Bax. 2005 Meeting on Programmed Cell Death, Cold Spring Harbor Laboratory, New York, USA.

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Abbreviations

2D	Two-dimensional
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid
Apaf-1	Apoptotic protease activating factor-1
ART	Apoptosis regulation of targeting domain
ATP	Adenosine triphosphate
Bax	Bcl-2 associated protein x
Bcl-2	B-cell lymphoma associated protein-2
BH	Bcl-2 homology domain
BH3	Bcl-2 homology domain-3
Bid	BH3-interacting domain death agonist
Ca^{2+}	Calcium ion
c-abl	Abl tyrosine kinase oncogene
CAD	Caspase-activated Dnase
CARD	Caspase activation and recruitment domain
Caspase	Cysteinyl aspartic protease
ced	Cell death abnormal gene
CED-3	Cell death abnormal protein-3
CED-4	Cell death abnormal protein-4
CED-9	Cell death abnormal protein-9
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid
c-myc	Myelocytomatosis cellular oncogene
CMV	Cytomegalovirus
Da	Daltons
kDa	Kilo Daltons
DED	Death-effector domain
DMSO	Dimethyl sulphoxide
DTT	dithiothreitol
EGL-1	Egg-laying defective-1
ER	Endoplasmic reticulum
FADD	Fas-associated death domain

µg	Microgram
mg	Milligram
HBSS	Hank's balanced salt solution
HRP	Horseradish peroxidase
Hsp	Heat shock protein
ICE	Interleukin-1β converting enzyme
IMM	Inner mitochondrial membrane
pI	Isolelectric point
IPTG	isopropyl-β-D-1-thiogalactopyranoside
µm	micrometer
ml	Millilitre
µl	Microlitre
LB	Luria-Bertani
MALDI	Matrix-assisted laser desorption/ionization
MALDI-TOF	MALDI - time-of-flight
mM	Millimolar
µM	Micromolar
MDM2	Mouse double minute protein-2
MPT	Mitochondrial permeability transition (pore)
Mr	Molecular weight
NGF	Nerve growth factor
nM	Nanomolar
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NP40	Nonidet P40
NPM	Nucleophosmin
OMM	Outer mitochondrial membrane
$P19^{ARF}$	Tumour suppressor protein p19 alternative reading frame
p53	Tumour suppressor protein p53
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline + 0.1% Tween-20
PCR	Polymerase chain reaction

PMSF	phenylmethylsulphonylfluoride
pRb	Retinoblastoma protein
psi	Pounds per square inch
RNAi	RNA interference
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
s.e.m	Standard error of the mean
shRNA	short hairpin RNA
siRNA	small interfering RNA
STS	Staurosporine
tBid	Truncated Bid
TMB	Tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TUNEL	terminal deoxynuceotidyl transferase-mediated dUTP nick end labelling
V	Volts
Vh	Voltage hours
VDAC	Voltage-dependant anionic channel
z-VAD.fmk	Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone

CHAPTER 1: General Introduction

1.1. Introduction

The multitude of cells that together form the tissue structures found within metazoan organisms are not immortal – the death of these cells is an inevitable and natural stage within their life cycle, and this loss, when accompanied by renewal, is an essential homeostatic mechanism central to the survival of all multi-cellular organisms, from worms to mammals. In a human, about a hundred thousand cells are produced every second by mitosis, and a similar number die by a physiological suicide process known as apoptosis (Vaux and Korsmeyer, 1999).

Apoptosis (from the Greek *apo* - from, *ptosis* – falling, literally refering to the seasonal falling of leaves from a tree or petals from a flower) is a co-ordinated process of cell self-destruction that is stereotyped by the fragmentation of nuclear DNA, chromatin condensation and the emergence of a distinctive cellular morphology leading to the eventual formation of membrane-bound apoptotic cell bodies that are finally engulfed by neighbouring phagocytes (Kerr *et al.*, 1972). Apoptosis is activated either by the presence of a death stimulus or by the removal of a survival factor (Duke and Cohen, 1986), and is a normal physiological process that is fundamental to developmental tissue remodelling and to the maintenance of tissue homeostasis throughout life (Bellamy *et al.*, 1995).

In 1972, researchers working at the University of Edinburgh published a seminal paper in which the term "apoptosis" was coined and which laid the foundation for the now widely accepted view that there are two physiologically-distinct mechanisms by which a cell can die: 1) necrosis, a passive, unregulated process characterised by cell lysis and the release of cellular contents into the extracellular matrix provoking an inflammatory response, and 2) apoptosis, a highly regulated, self-programmed process leading to the removal of damaged or ectopic cells in the absence of inflammation (Kerr *et al.*, 1972) -

see Figure 1.1 for a comparison of the morphological differences between these two models of cell death. Necrotic, or lytic, cell death is usually confined to the aftermath of acute cell injury or trauma, such as that resulting from hypoxia, heat shock, viral invasion or exposure to various exogenous toxins, and is associated with severe pathophysiology, principally as a consequence of subsequent inflammation and secondary tissue damage (Leist and Nicotera, 1997). Apoptosis, in contrast, is a programmed event dependent on continued ATP synthesis (Leist *et al.*, 1997) by which superfluous cells are removed during development, particularly within the immune and nervous systems, as well as mediating the elimination of damaged or ectopic cells throughout the course of normal tissue homeostasis within the adult organism (Steller, 1995; Adams, 2003). However, apoptosis, when inappropriately activated, is also known to be a major contributor to cell pathology associated with numerous disease states, including AIDS (Gougeon *et al.*, 1993), myelodysplastic syndromes (Yoshida, 1993), myocardial and cerebral infarction (Charriaut-Marlangue *et al.*, 1996; Olivetti *et al.*, 1997; Hara *et al.*, 1997), and the neurodegenerative disorders, Alzheimer's disease (Loo *et al.*, 1993; Cotman and Anderson, 1995), Parkinson's disease (Jenner and Olanow, 1998), Huntington's disease (Ona *et al.*, 1999) and amyotrophic lateral sclerosis (ALS) (Kostic *et al.*, 1997). Conversely, pathophysiology associated with dysfunctional apoptosis includes chronic inflammation and autoimmunity, due to the impaired clearance of pro-inflammatory cells (Gilroy *et al.*, 2004); the replication and pathogenesis of oncogenic viruses, such as Epstein Barr virus (EBV) (Henderson *et al.*, 1993), human cytomegalovirus (CMV) (Goldmacher *et al.*, 1999) and hepatitis B virus (Bellamy *et al.*, 1995); and tumourogenesis (Tsujimoto *et al.*, 1985; Hollstein *et al.*, 1991). Thus, the extent of apoptosis within a multi-cellular organism is a finely controlled process: too much results in excessive cell pathology whereas too little can set the scene for cancer to take hold. Only by improving our understanding of the molecular pathways leading to apoptosis can we stand a chance of developing therapeutics that will allow us to manipulate the course of apoptosis to avoid either of those two outcomes. The relevance of apoptosis to current medical research is perhaps best illustrated by the exponential increase in the number of research papers that refer to

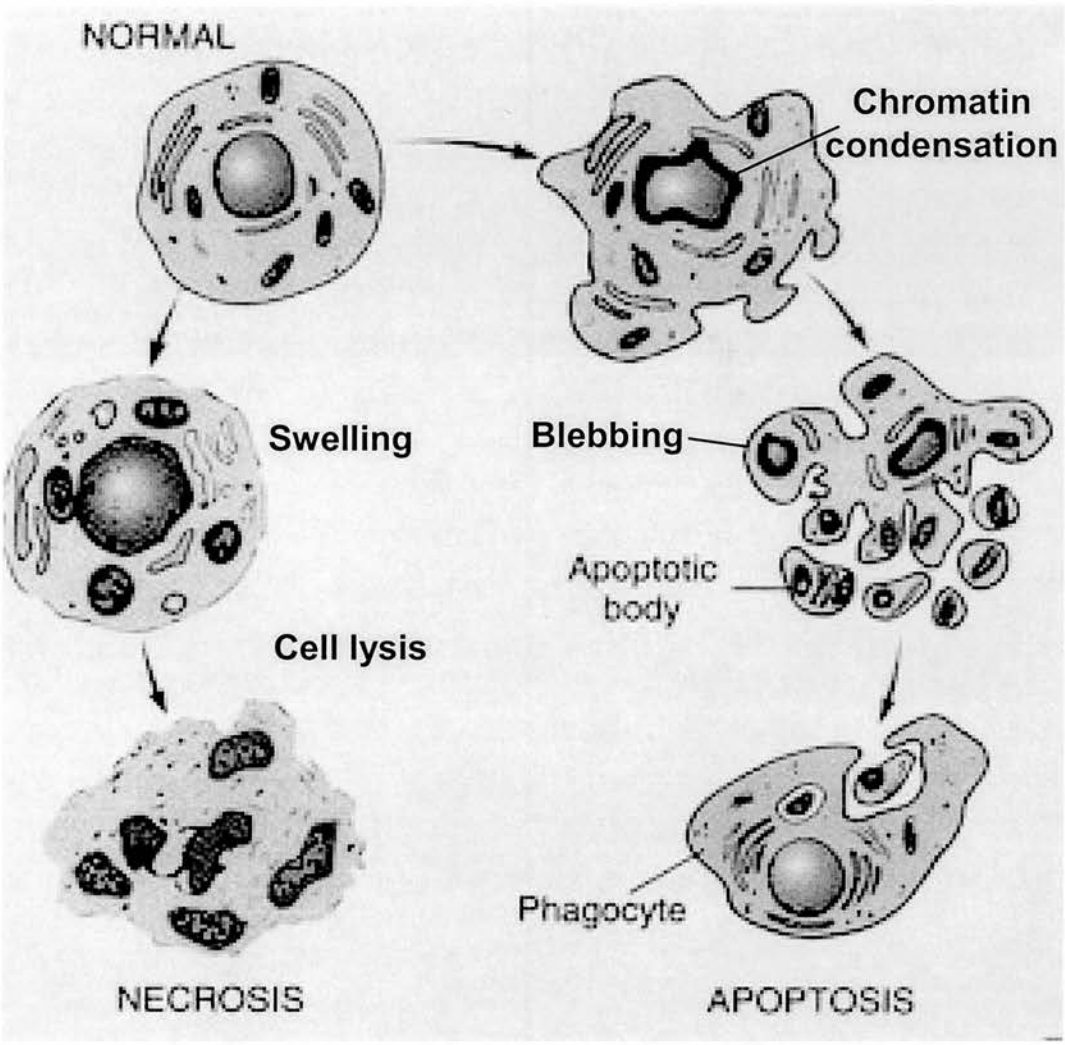

Figure 1.1. Differentiating between apoptosis and necrosis. Necrotic cell death is characterised by the swelling of the cell and organelles, the appearance of mottled chromatin condensation and loss of membrane asymmetry. The final outcome is lysis of the plasma and intracellular membranes with the release of cellular contents, including proteases and lysozymes, into the extra-cellular environment triggering an inflammatory response. Apoptosis begins with cell rounding, shrinkage of the cell volume and compaction of the intracellular environment. The nucleus condenses and chromatin marginalises to the nucelar envelope where it forms dense granular caps. The fragmentation of the nucleus into membrane-bound vesicles is accompanied by blebbing of the plasma membrane (zeiosis). The entire cell then splits into multiple membrane-bound "apoptotic bodies" which are then rapidly engulfed by neighbouring phagocytes without eliciting an inflammatory response.

"apoptosis" published in the past two decades – see Figure 1.2. As a result of this intensive research, our understanding of the complexities involved in apoptotic signalling has improved dramatically. However, despite this, a great deal of uncertainty and controversy in the interpretation of research findings persists throughout the cell death community.

The experiments presented within this thesis were performed within the Astellas CNS Research in Edinburgh (ACE) laboratories (formerly known as the Fujisawa Institute for Neuroscience in Edinburgh (FINE) until 2004). The research focus of ACE, and therefore of this thesis, lies within neuronal cell death, in particular the contribution made by apoptosis to the pathophysiology of cerebral ischaemic cell death and neurodegeneration. Apoptosis can be initiated by many signals in neuronal cells. Stimuli associated with neurodegenerative disorders include lack of trophic factor support, release of excitotoxic amine neurotransmitters and metabolic stress (Thompson, 1995; Mattson, 2000). Central to the apoptotic pathway initiated by such stressors is mitochondrial dysfunction (Kroemer *et al.*, 1998) which leads to the release of apoptogenic molecules and the execution of apoptosis. The transmission of the apoptotic signal to the mitochondrial surface is regulated by the B-cell lymphoma protein-2 (Bcl-2) family of proteins, of which the pro-apoptotic protein, Bcl-2 associated X protein (Bax), is a pivotal member. The relocalisation of Bax from the cytosol to the mitochondrial surface is an early event within neuronal cell apoptosis (McGinnis *et al.*, 1999; Putcha *et al.*, 1999), and the deletion of Bax has been shown to protect sympathetic neurones against NGF-withdrawal-induced apoptosis (Deckwerth *et al.*, 1996). Identifying the regulatory factors controlling Bax activation within neuronal cells is therefore the principle aim of this thesis, the results of which are presented within the following chapters. However, apoptosis is a physiologically important process in all tissues during development and throughout life and is therefore associated with the emergence of a multitude of disease states and pathologies when deregulated. The remainder of this introductory chapter will therefore provide an overview of the physiological processes in which apoptosis contributes, the molecular pathways

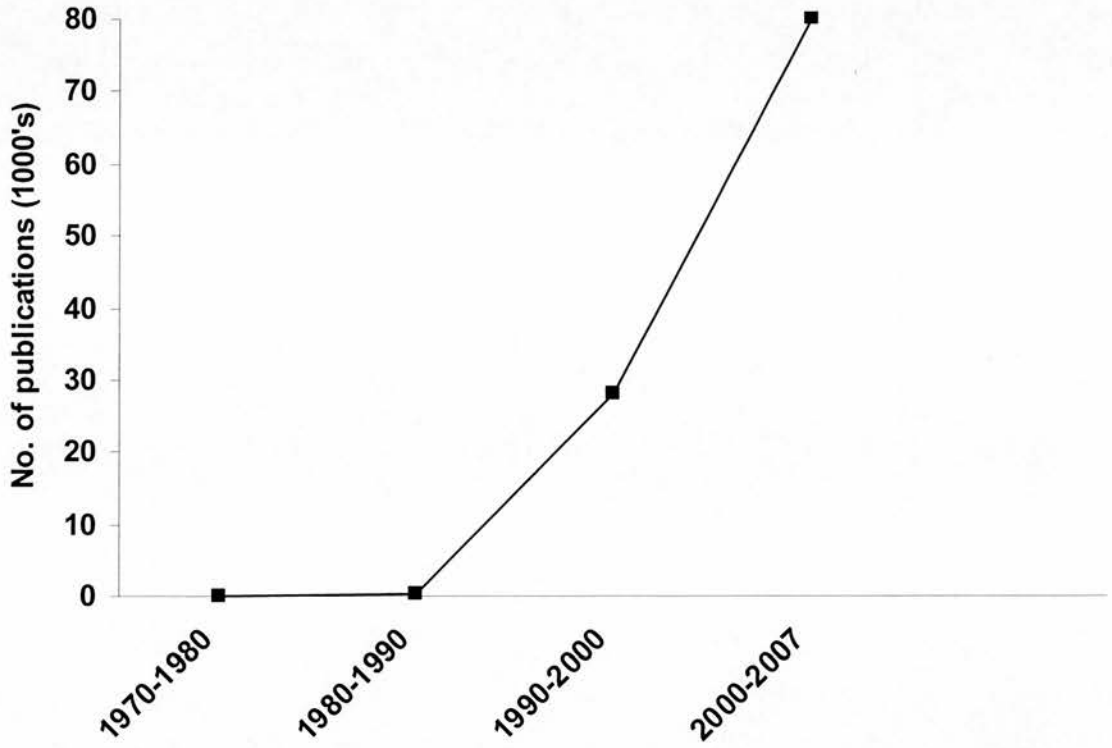

Figure 1.2. World-wide research publications on apoptosis. Research papers on apoptosis remained relatively obscure even up to the late 1980's with on average 10 papers published a year. Thereafter, the rate of publication and interest has risen exponentially, with currently over 200 publications appearing every week that refer to "apoptosis". Data compiled using www.pubmed.gov.

involved and an in-depth discussion on the structure and function of Bax within neuronal cell death pathways.

1.2. Apoptosis during development

Before the concept of apoptosis had arisen, developmental biologists had for a long time been observing the stereotyped "programmed" phases of cell death known to occur during the development of many organisms, including insects (Lockshin and Williams, 1965), nematodes (Ellis and Horvitz, 1986) and amphibians (Tata, 1966). Indeed, this process of naturally occurring cell death, now morphologically, biochemically and genetically recognisable as apoptosis, is highly conserved amongst all metazoan organisms and is essential for the developmental maturation of tissues (Vaux and Korsmeyer, 1999).

During mammalian development, apoptosis is involved in the formation of channels and cavities within the central nervous system, the removal of interdigital webbing, the remodelling of bone and the involution of the mammary glands (Vaux and Korsmeyer, 1999). Furthermore, the production of excess cells and the subsequent removal of superfluous cells by apoptosis is a feature common to the development of many types of cells, tissues and organs (Oppenheim, 1991; Vaux and Korsmeyer, 1999; Opferman and Korsmeyer, 2003), and probably exists to ensure that the optimal number of cells are available for later demands (Oppenheim, 1991).

Studies using gene knock-out mice revealed that apoptosis plays a central role in the developing nervous system (Motoyama *et al.*, 1995; Kuida *et al.*, 1996; Hakem *et al.*, 1998; Yoshida *et al.*, 1998). For example, transgenic mice lacking expression of the apoptogenic enzymes, caspase-3 and caspase-9, both key components of apoptotic pathways, suffered excessive brain growth and neurodevelopmental abnormalities as a result of reduced apoptosis and died perinatally (Kuida *et al.*, 1996; Hakem *et al.*, 1998). Similarly, deletion of another key pro-apoptotic protein, Bax, resulted in the abolishment

of developmental cell death occurring in sympathetic and motor neuronal cell populations, although these mice remained viable into adulthood (Deckwerth *et al.*, 1996; White *et al.*, 1998). Such a reliance by the nervous system on apoptosis is due in part to the extensive remodelling of the central and peripheral nervous system structures that occurs during embryonic and postnatal developmental periods (Oppenheim, 1991). This example of so-called "morphogenetic" cell death, whereby tissue structures are sculpted by the selective removal of excess cells, is widely observed throughout development but is particularly pertinent to the mammalian nervous system where the formation of various cavities and channels is essential for its function (Oppenheim, 1991).

Development of the nervous system, as with other tissues, such the immune and haematopoietic systems, is characterised by massive cell death (ranging from 20-80% depending on the neuronal cell population) occurring towards the latter stages of the maturation process (Glucksmann, 1951; Oppenheim, 1991). Furthermore, this extensive cell death appears to involve almost all neuronal types and is prevalent in both the central and peripheral nervous systems (Oppenheim, 1991). Afflicted neurones are post-mitotic and display the phenotypic characteristics of their given cell type, but have failed to establish post-synaptic connections with the appropriate afferent cells (Oppenheim, 1991; Sadoul, 1998). Elimination of these cells resembles the positive selection process seen during the thymic maturation of T cells whereby cells die "by neglect". In the case of neurones, this is mediated by the withdrawal of neurotrophic factors, such as nerve growth factor (NGF), leading to the inability of these cells to counteract intrinsic apoptotic signalling pathways (Deckwerth and Johnson, Jr., 1993; Edwards and Tolkovsky, 1994; McKay *et al.*, 1999).

1.3. Apoptosis in adult physiology

Once the development of cell lineages is complete, the maintenance of cell number as well as the viability of the adult organism depends on attaining a balance between

proliferation (mitosis) and cell death (apoptosis). Each of these two essential physiological processes is under genetic control – cell proliferation is governed by growth factors and proto-oncogene expression (Rozengurt, 1992) whereas cell death is initiated by the removal of survival factors and the expression of tumour suppressor genes (Levine, 1993).

Aside from the general maintenance of cellular homeostasis, apoptosis also performs several well defined roles in physiological processes, particularly within the immune system. During an immune response, there is a rapid clonal expansion of antigen-specific lymphocytes that is sustained by the expression of pro-survival cytokines and anti-apoptotic proteins (June *et al.*, 1987; Boise *et al.*, 1995). However, once the immune threat has subsided, the large population of now redundant post-activation lymphocytes is selectively eliminated by apoptosis through the combined effect of reducing expression of anti-apoptotic proteins, increasing expression of pro-apoptotic proteins and the activation of cell surface death receptors (Opferman and Korsmeyer, 2003). Similarly, the clearance of potentially harmful pro-inflammatory cells, such as neutrophils, eosinophils and monocytes, from sites of inflammation during the latter stages of the immune response is controlled by apoptosis (Bellamy *et al.*, 1995) as is the removal of autoimmune T cells that can arise as a result of somatic mutation during an immune response (Fisher *et al.*, 1995; Borner, 2003).

In contrast to the rapid turnover of cells in proliferative tissues, and to the extensive remodelling during development, naturally occurring cell death within the adult nervous system is much less apparent due to the limited capacity for self-renewal exhibited by mature neural cells and their resultant long life span (Thompson, 1995; Mattson, 2000). However, as will be discussed in the following section, neurones are particularly sensitive to a variety of stress stimuli, including ischaemic, excitotoxic and oxidative injury where apoptosis, alongside necrosis, accounts for a significant proportion of neuronal cell loss.

1.4. Apoptosis in pathology

As with many essential physiological processes, deregulation of apoptosis can lead to the emergence of severe disease/pathology (Thompson, 1995). The diseases/pathologies resulting from deregulated apoptosis are either a consequence of excessive or inappropriate activation, such as that associated with AIDS, myocardial and cerebral infarction, ischaemia-reperfusion damage and neurodegenerative diseases (Thompson, 1995; Olivetti *et al.*, 1997; Mattson, 2000; Rathmell and Thompson, 2002), or when apoptosis is non functional due to gene mutation which can lead to auto-immunity, excess inflammation and tumour formation (Fisher *et al.*, 1995; Zornig *et al.*, 2001; Green and Evan, 2002) – see Table 1.1.

1.4.1. Apoptosis and neurological disorders

The reliance of the nervous system on apoptosis during development is equalled by its vulnerability to disruption when neuronal apoptotic pathways become overwhelmed and inappropriately utilised during severe insults, such as those associated with stroke and traumatic brain and spinal cord injury (Yakovlev *et al.*, 1997; Emery *et al.*, 1998; Dirnagl *et al.*, 1999). Apoptosis has also been linked with the prolonged attrition of neuronal cells during the course of chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and ALS (Thompson, 1995; Mattson, 2000). Apoptosis in these neurological disorders can be initiated by numerous physiological triggers, including lack of neurotrophic factor support (McKay *et al.*, 1999), excitotoxicity (Choi, 1992; Ankarcrona *et al.*, 1995; Wong *et al.*, 1998) and oxidative stress (Sastry and Rao, 2000).

The devastating physical and mental manifestations accompanying the progression of neurodegenerative diseases originate from the death of specific neuronal populations within the nervous system (Thompson, 1995). For example, death of hippocampal neurones underlies some of the cognitive defects associated with Alzheimer's disease

Diseases associated with the inhibition of apoptosis	Diseases associated with increased apoptosis
Cancer	**AIDS**
Follicular lymphoma	**Neurodegenerative disorders**
Carcinomas with *p53* mutations	Alzheimer's disease
Hormone-dependant tumours	Parkinson's disease
Breast cancer	Huntington's disease
Prostate cancer	Amyotrophic lateral sclerosis
Ovarian cancer	**Ischemic injury**
Autoimmune disorders	stroke
Systemic lupus erythematosus	myocardial infarction
Immune-mediated glomerulonephritis	reperfusion injury
Autoimmune lymphoproliferative syndrome	**Myelodysplastic syndromes**
Viral infections	Aplastic anemia
Herpesviruses	**Toxin-induced liver disease**
Poxviruses	Alcohol
Adenoviruses	

Table 1.1. Diseases associated with deregulated apoptosis.

(Cummings *et al.*, 1998); the loss of dopaminergic neurones in the substantia nigra underlies the uncoordinated movements characterising Parkinson's disease (Jenner and Olanow, 1998); Huntington's disease is associated with the destruction of neurones in the striatum that control body movements (Ona *et al.*, 1999); and the loss of lower motor neurones leads to the disabling physical prognosis faced by patients with ALS (Martin *et al.*, 2000). In each case, the activation of apoptotic death pathways in afflicted neurones has been proposed to be a major contributor to cell loss and disease progression (Thompson, 1995; Mattson, 2000).

The destructive neuronal cell damage and death resulting from stroke has also been observed to involve a significant apoptotic component (Mattson *et al.*, 2001). Histochemical analysis of rodent brains removed from experimental focal ischemic stroke models revealed a central necrotic "core", in which neurones die rapidly (within minutes to hours) by necrosis, and a surrounding sub-optimally perfused "penumbra" in which glial cells survive but damaged neurones die over a protracted period lasting several days (Dirnagl *et al.*, 1999; Mattson *et al.*, 2001; Plesnila, 2004). Biochemical analyses of post-mortem tissue extracted from the ischemic penumbra of stroke patients (Guglielmo *et al.*, 1998) and rat models of focal cerebral ischaemia (Linnik *et al.*, 1993; Fujimura *et al.*, 1998) have demonstrated the occurrence of widespread apoptotic neuronal cell death in this region. Over time, the penumbral tissue degenerates to necrotic infarct tissue, in which all neuronal cell-types die. Hence, anti-apoptotic pharmacological intervention within the appropriate therapeutic window (a few hours) following stroke could potentially lead to the recovery of neuronal cell death within this region.

As an illustration of the prevalence of apoptosis in neurological disorders, the presence of apoptogenic active caspases has been observed in Alzheimer's disease (Gervais *et al.*, 1999; Chan *et al.*, 1999; LeBlanc *et al.*, 1999), Parkinson's disease (Hartmann *et al.*, 2000; Tatton, 2000), Huntington's disease (Sanchez *et al.*, 1999), ALS (Pasinelli *et al.*, 1998), as well as in the neuronal cell damage associated with stroke (Namura *et al.*,

1998) and spinal cord injury (Matsushita *et al.*, 2000). Furthermore, pro-apoptotic proteins belonging to another major group of apoptosis-regulating proteins, the Bcl-2 family of proteins, have also been implicated in contributing to the neurpathological cell death associated with such disorders (Plesnila, 2004; Akhtar *et al.*, 2004; Ouyang and Giffard, 2004). Thus, alleviating apoptotic neuronal cell death by therapeutic intervention could make a significant improvement to the prognosis faced by patients with these disorders.

1.4.2. Apoptosis and cancer

It is a widely observed phenomenon that cells associated with many types of human malignancies are resistant to apoptosis (Hoffman and Liebermann, 1994). Normal cells are dependent on survival factors for continued growth and undergo apoptosis when such factors are removed either as a result of growth factor withdrawal (Duke and Cohen, 1986; Williams *et al.*, 1990; Raff *et al.*, 1993) or when a cell becomes displaced from its physiological setting (Boudreau *et al.*, 1995; Valentijn *et al.*, 2004). Metastatic tumour cells that have defective apoptosis-promoting genes are able to circumvent this latter homeostatic restriction to relocate to distinct tissue sites and proliferate (Thompson, 1995).

The observed immortality of many tumour cells is known to arise from genetic mutation within several key apoptosis-regulating genes. The first of these to be identified was oncogenic *bcl-2* as a result of its 14;18 translocation and over-expression in human follicular lymphoma (Tsujimoto *et al.*, 1984; Cleary *et al.*, 1986). Although originally identified as an oncogene, over-expression of the *bcl-2* gene product was later found to promote the clonal expansion of tumour cells by inhibiting apoptosis (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990). Aside from lymphomas, over-expression of Bcl-2 protein is known to underlie the propagation of several other human cancers, among them prostatic (McDonnell *et al.*, 1992), colorectal (Hague *et al.*, 1994) and neuroblatomas (Castle *et al.*, 1993). Other anti-apoptotic Bcl-2 family proteins are also potential oncogenes,

however so far little evidence is available that directly links them to the emergence of specific tumour types (Cory *et al.*, 2003). In contrast, pro-apoptotic proteins within the Bcl-2 family represent potential tumour suppressors, and loss of function of any of these proteins could conceivably lead to tumourogenesis. For example, the loss of pro-apoptotic Bax function is associated with some human gastrointestinal cancers (Rampino *et al.*, 1997) and leukemias (Meijerink *et al.*, 1998), and mutation of the BH3-only protein, Bim, which plays a key role in regulating lymphoid homeostasis (Bouillet *et al.*, 1999), represents a candidate genetic basis for the emergence of human leukemias, although this has yet to be proven (Cory *et al.*, 2003). However, a tumour suppressor that is known to be a critical factor in many human cancers is the transcriptional regulator, p53. In response to genotoxic damage, the upregulation of p53 protein leads to the initiation of apoptosis through the transactivation of pro-apoptotic genes, including pro-apoptotic Bcl-2 family proteins (Lowe *et al.*, 1993; Haupt *et al.*, 2003). Tumour cells deficient in p53 therefore display enhanced resistance to DNA damage induced by radiation and chemotherapeutic drug treatments (Lowe *et al.*, 1994). Indeed, direct gene mutation of *p53* underlies almost half of all human malignancies (Vogelstein *et al.*, 2000), and a proportion of the remaining tumours are associated with elevated levels of p53-inhibiting proteins, such as MDM2 (Evan and Littlewood, 1998), or mutations to p53 synergistic proteins, such as the $p19^{ARF}$ tumour suppressor (Haber, 1997). Together, these observations demonstrate the pivotal role that apoptosis plays in protecting metazoan organisms from the emergence and clonal expansion of malignant cells.

1.5. Elucidating the molecular pathways of apoptosis

From pioneering genetic studies carried out in the 1980s by H. Robert Horvitz and colleagues using the nematode *Caenorhabditis elegans*, it was demonstrated that the molecular events leading to apoptosis were genetically pre-determined (Horvitz et al, 1982; Ellis & Horvitz, 1986). Of the 1090 somatic cells within the hermaphrodite worm, 131 are destined to die during development in a precisely co-ordinated manner (Ellis *et*

al., 1991). Detailed microscopic observation of the morphology of the dying cells confirmed that they were undergoing apoptosis, with their complete degradation and engulfment by neighbouring cells taking place within one hour. This process was found to be regulated by a number of genes known as *ce*ll *d*eath abnormal (ced) genes, the first to be discovered being *ced-3* and *ced-4* which were found to be required for the initiation of programmed cell death during this developmental period (Ellis and Horvitz, 1986). Within the mammalian system, the first gene to be identified as a key regulator of apoptosis initiation was *bcl-2* (Vaux *et al.*, 1988). *Bcl-2* was originally identified in 1984 as a gene activated as a result of a chromosomal translocation event (t14;18) in human follicular lymphoma (Tsujimoto *et al.*, 1984). However, the possibility that *bcl-2*, in contrast to other oncogenes involved in translocations known at that time, such as *c-abl* and *c-myc*, could also inhibit cell death pathways rather than just promote cell proliferation did not emerge until several years later when its over-expression was found to prevent cells from dying when growth factor was removed (Vaux *et al.*, 1988). This discovery, together with the realisation that p53, one of the most frequently mutated genes in cancer, was a key pro-apoptotic gene (Yonish-Rouach *et al.*, 1991) and that the apoptotic pathway initiated by p53 can be blocked by Bcl-2 (Chiou *et al.*, 1994) reinforced the regulatory role of Bcl-2 in apoptosis and established the profoundly significant link between the inhibition of apoptosis and the development of cancer in humans (Vaux, 2002). Furthermore, in 1992, definitive proof that programmed cell death in *C.elegans* and apoptosis in mammals is the same evolutionary conserved physiological process was provided by showing that the human *bcl-2* gene could inhibit developmental cell death when expressed in the worm (Vaux *et al.*, 1992). In the same year, another *C.elegans ced* gene was discovered, *ced-9*, that was found to inhibit the pro-apoptotic influences of CED-3 and CED-4 (Hengartner *et al.*, 1992) through direct interaction with CED-4 on the mitochondrial surface (Chen *et al.*, 2000). CED-9 was later determined to be a homolog of the mammalian Bcl-2 protein (Hengartner and Horvitz, 1994). As comprehension of the molecular events within mammalian apoptosis burgeoned, other relationships linking invertebrate and vertebrate apoptotic pathways emerged. In 1993, Yuan and colleagues published their observation that a similarity

existed between the nematode *ced-3* gene product and the mammalian interleukin-1β converting enzyme (ICE) (Yuan *et al.*, 1993), at the time recently discovered to be a unique cysteine protease involved in the maturation of interleukin-1β to its active pro-inflammatory, biologically-active form (Thornberry *et al.*, 1992; Cerretti *et al.*, 1992). ICE, now known as caspase-1, was the first of a family of mammalian cysteinyl proteases to be characterised that together orchestrate the dismantlement of the apoptotic cell through the cleavage of essential cellular substrates. Furthermore, when the mammalian caspase-activating adapter protein, apoptotic protease activating factor-1 (Apaf-1) was identified in 1997, it was found to be homologous to CED-4 (Zou *et al.*, 1997). To complete the unification of these two cell death pathways, the *C.elegans* protein, EGL-1 (*eg*g-*l*aying defective-1) was identified in 1998, again by researchers in the Horvitz laboratory, and was found to interact with and inhibit the protective protein CED-9 (Conradt and Horvitz, 1998) – see Figure 1.3. Structural analysis of this novel protein demonstrated that it shared sequence homology with only one region within both CED-9 and Bcl-2 known as the BH-3 domain and represented the nematode equivalent of a diverse group of pro-apoptotic signalling proteins active in mammalian apoptosis known as the BH3-only proteins (Conradt and Horvitz, 1998).

Despite this apparent unification of invertebrate and vertebrate apoptotic pathways, the mammalian apoptotic mechanism is in fact a great deal more complex as it offers an alternative extrinsic death-receptor-initiated pathway to the more ancient and evolutionary conserved intrinsic, or mitochondrial, pathway, as will be described in more detail in the next section of this introduction. Furthermore, due to the propensity for increasing complexity as one ascends the phylogenetic tree, mammalian apoptosis is orchestrated by a multitude of regulatory proteins in contrast to the relatively simplistic pathway operating within *C.elegans*.

In addition to the intrinsic and extrinsic pathways, apoptosis can also be initiated within the mammalian system by the release of granzyme B from cytotoxic T and NK cells (Shi *et al.*, 1996) and an endoplasmic reticulum (ER)-directed pathway in response to

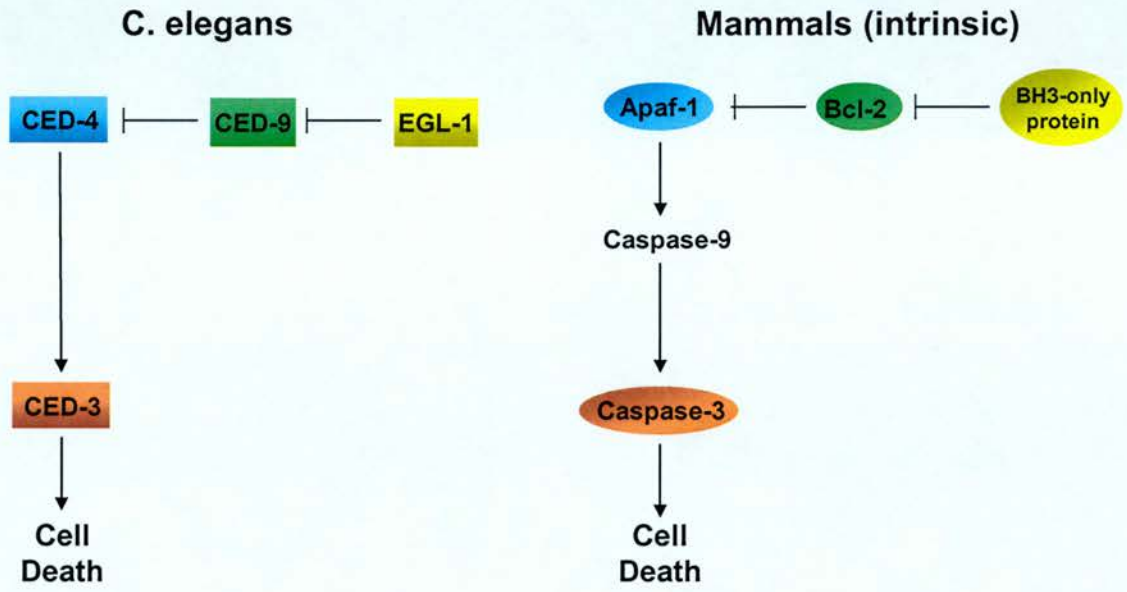

Figure 1.3. Comparison between nematode and mammalian apoptotic cell death pathways. Programmed cell death (apoptosis) in the nematode, C. elegans, requires CED-4 and CED-3. CED-9 interacts directly with CED-4 and both are thought to reside on mitochondria. In cells destined to die during development, expression of the death-promoting protein, EGL-1 binds to CED-9 and displaces it from CED-4 which then initiates apoptosis via CED-3. In the mammalian system, the recruitment of Apaf-1 is inhibited by the CED-9 homolog, Bcl-2, which prevents cytochtrome c from being released from mitochondria. Bcl-2 is in turn inhibited by pro-apoptotic BH3-only proteins that represent the mammalian homolog of EGL-1. Apaf-1 promotes the activation of the initiator caspase, caspase-9 which in turn activates the CED-3 homolg and executioner caspase, caspase-3. Apoptotic cell death then ensues.

perturbed intracellular Ca^{2+} homeostasis or the accumulation of misfolded proteins in the cytosol and/or ER lumen (Zinszner *et al.*, 1998; Nakagawa *et al.*, 2000). However, there exists a degree of overlap between all of these routes and most pathways are united in their latter stages through the activation of caspases. These enzymes, as will be expanded upon in the following section, account for the characteristic morphological and biochemical hallmarks associated with apoptosis, such as nuclear fragmentation, chromatin condensation, cell rounding, shrinkage and plasma membrane blebbing (zeiosis) (Wyllie *et al.*, 1980).

The stereotypical sequence of morphological changes exhibited by cells undergoing apoptosis is directed by a tightly regulated intracellular signalling cascade involving death receptors on the plasma membrane, signal transducing molecules, cytosolic and membrane-bound proteins, proteolytic enzymes and transcription factors. The molecular pathways to apoptosis can be broadly delineated into three main phases: the initiation, execution and termination phases (Schmitz *et al.*, 2000). In this model, apoptosis is initiated by numerous death signals, including withdrawal of survival factors ("death by neglect"), irradiation, hypoxia, chemotherapeutic agents and ligation of death receptors. The death signal is then relayed throughout the cell death machinery leading to the activation of dormant pro-apoptotic proteins and the suppression of survival factors, for example those belonging to the Bcl-2 protein family. The execution phase is choreographed by the caspase family of proteases that systematically cleave numerous cellular substrates to bring about the careful dismantlement of the cellular environment (Thornberry and Lazebnik, 1998). Finally, in the termination phase, membrane-bound cellular material is engulfed by phagocytes (Savill *et al.*, 1993).

1.6. Caspases

Caspase activation is a biochemical hallmark of apoptosis (Abraham and Shaham, 2004). When active, this family of proteases targets essential cellular substrates, among them key structural proteins, such as the actin-regulatory protein, gelsolin, and the

cytoskeletal protein, α-fodrin, which may account for some of the morphological changes seen in apoptosis, such as zieosis and cell rounding/shrinkage (Martin *et al.*, 1995; Janicke *et al.*, 1998b). Caspases are also known to have activity within the nucleus where they activate DNases, such as DNA fragmentation factor 45kDa subunit (DFF-45)/caspase-activated DNase (CAD), which cleave genomic DNA into ~180bp inter-nucleosomal fragments (Tang and Kidd, 1998), one of the key determinants of apoptotic cell death.

The caspase family are *c*ysteine proteases that cleave their substrates after an *asp*artate residue (hence their name), a target site shared by the serine protease, granzyme B, but otherwise unique amongst mammalian enzymes (Degterev *et al.*, 2003). The caspase target sites within substrate polypeptides consist of a short tetrapeptide motif that all begin with an aspartate residue (Nicholson, 1999). However, different caspase family members exhibit a preference for specific residues within the remaining three positions, a promiscuity that has enabled the generic classification of the caspases into three subgroups according to their substrate specificity (Thornberry *et al.*, 1997). This categorisation led to the assignment of several important functionalities to caspase groups. For example, Group III caspases, such as caspases-3 & -7, were found to share a highly stringent target site sequence (DEXD, where X is either V, T or H) that was also recognised by CED-3 and appeared in many proteins cleaved during apoptosis (Nicholson, 1999). These caspases therefore represented the "effector" caspases within the family (Thornberry *et al.*, 1997). In contrast, caspases belonging to group II , which included caspases-8, -9 & -10, were found to preferentially cleave a target site found on effector caspases, which implicated them as upstream "initiator" caspases that activated the effector caspases in response to apoptotic signalling (Thornberry *et al.*, 1997). Group I caspases, represented by caspases-1, 4 and 5, are also initiator caspases but exhibit a dual role within apoptosis and inflammation where they are required for the proteolytic maturation of pro-inflammatory cytokines, such as pro-interleukin 1β, interleukin-18 and pro-interferon (IFN)γ inducing factor (Kuida *et al.*, 1995; Li *et al.*, 1995; Thornberry *et al.*, 1997). Classifying caspases according to structure was also

found to result in similar grouping as those above, suggesting a close structure-function relationship within the caspase family. For example, caspases belonging to initiator class were found to contain extended prodomain regions that facilitated interaction with upstream signalling pathways (Degterev *et al.*, 2003). Furthermore, the prodomain region of latent initiator caspases (known as procaspases) were found to contain one of two characteristic protein-protein binding motifs – the death effector domain (DED), found in caspases-8 and -10, and the caspase activation and recruitment domain (CARD) present in caspase-9, as well as caspases-1, -2, -4 and -5, (Degterev *et al.*, 2003). The DED and CARD motifs were shown to be recognised by two different adaptor proteins, each specifically associated with the two main pathways to apoptotic cell death in mammalian cells - the extrinsic and intrinsic pathways, respectively. For example, the death receptor component within the extrinsic pathway, Fas-associated death domain (FADD), binds to the DED on procaspases-8 and -10 (Muzio *et al.*, 1996), and the intrinsic pathway-associated component, Apaf-1, forms a complex with procaspase-9 through its binding to CARD (Rodriguez and Lazebnik, 1999). This selective activation of initiator caspases is a molecular hallmark that can be used to differentiate between an extrinsic or intrinsic route to apoptosis – see Figure 1.4. However, as will be discussed later, there is often a high degree of overlap between these two pathways that can make such observational rules of thumb misleading.

Initiator caspases selectively activated by the extrinsic and intrinsic pathways converge upon the effector, or "executioner", caspases, comprising caspases-3, -6 and -7. The effector caspases, like the initiator caspases, are present in the resting cell as latent procaspase zymogens, and require internal cleavage by initiator caspases to become fully activated (Thornberry *et al.*, 1997; Nicholson, 1999). However, once in their activated form, the effector caspases proteolytically cleave numerous protein substrates, amongst them nuclear factors (for example caspase-activated DNase (CAD) (Tang and Kidd, 1998), cytoskeletal proteins (for example α-fodrin (Janicke *et al.*, 1998a)) and signalling molecules, such as protein kinases, to bring about apoptotic cell death (Degterev *et al.*, 2003).

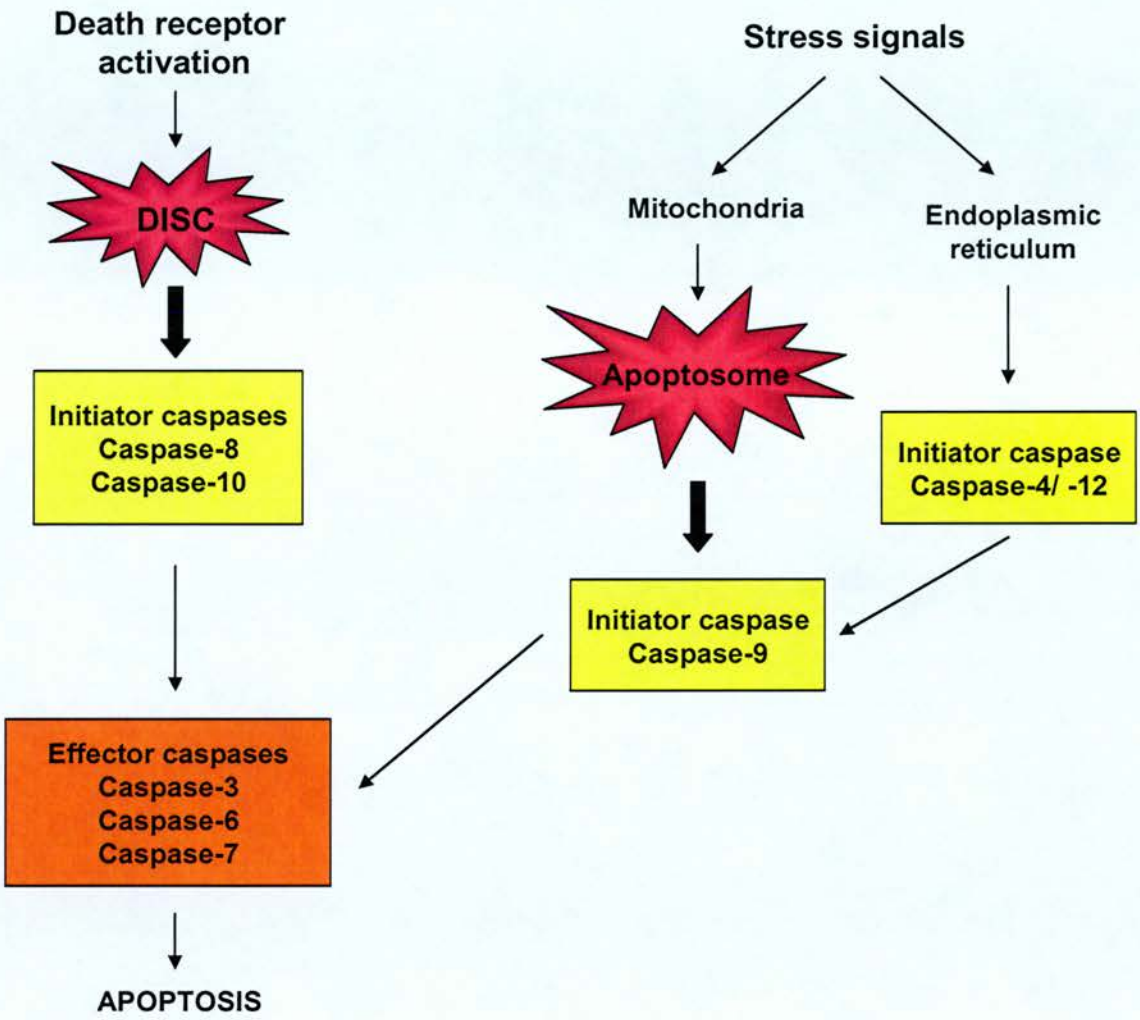

Figure 1.4. Caspase activation pathways. Two ATP-dependent catalytic caspase-generating complexes are initiated in response to either extrinsic or intrinsic stimuli. The highly homologous caspases-8 and -10 are generated by the death-inducing signalling complex (DISC) in response to death receptor ligation and directly activate executioner caspases, -3, -6 and -7. In response to cellular stress, release of apoptogenic factors from mitochondria leads to the formation of the apoptosome, whereas ER stress activates caspase-12 (in the mouse) and perhaps the human homolog, caspase-4. Both mitochondria and ER routes lead to the activation of caspase-9 which then activates the executioner caspases, -3, -6 and -7.

1.7. The extrinsic and intrinsic pathways to apoptosis

As has already been mentioned, two main routes to caspase activation have been characterised in the mammalian system: the extrinsic pathway, which relies on the activation of cell-surface death receptors, and the more ancient and evolutionary conserved intrinsic pathway that is activated by a variety of cellular stresses and involves the release of apoptogenic molecules from within mitochondria (Adams, 2003). Although both pathways are triggered by distinct stimuli and have the capacity to initiate caspase activity independently from each other (Strasser *et al.*, 1995), it has become apparent that under many circumstances both pathways cooperate in order to maximise the rate of caspase activation within any given cell undergoing apoptosis (Schmitz *et al.*, 2000; Degterev *et al.*, 2003). An overview of the intrinsic and extrinsic pathways to apoptotic cell death is provided in Figure 1.5.

1.7.1. The extrinsic (or death-receptor) pathway

The extrinsic apoptotic pathway is initiated when death ligands bind to corresponding death receptors on the extracellular surface of the plasma membrane on the cell destined to die (Ashkenazi and Dixit, 1998). This type of "instructive" apoptosis is particularly important within the immune system where cytotoxic T cells expressing death ligands on their surface engage virus-infected and/or cancerous cells and target them for destruction by apoptosis (Boise and Thompson, 1996). The elimination of redundant immune and inflammatory cells at the end of the immune response, as well as the destruction of auto-reactive T- and B-cells, is also mediated by the engagement of death receptors and the initiation of the extrinsic apoptotic pathway (Osborne, 1996), preventing excessive cytokine release and autoimmunity that may otherwise result in extensive tissue damage.

Of the six death receptors currently identified, Fas/CD95, TNF-R1 and TRAIL-R1 and –R2 receptors are the best characterised. These receptors are type-I transmembrane

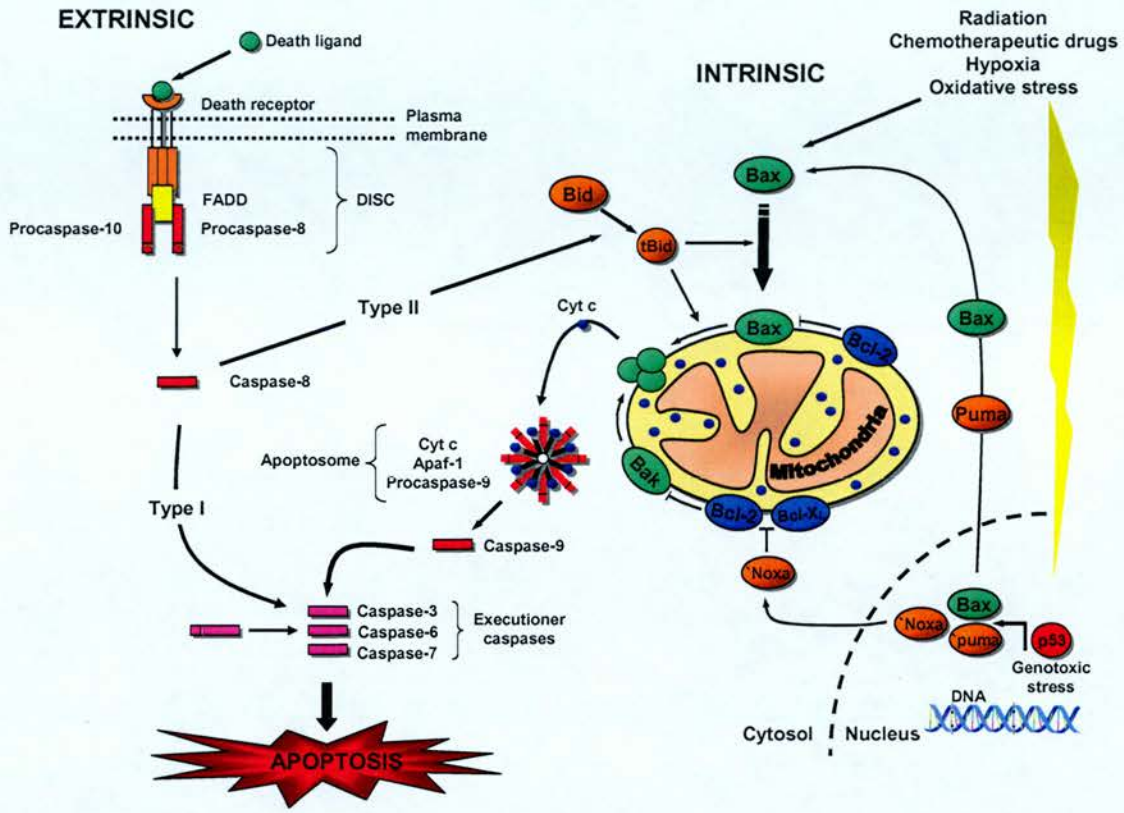

Figure 1.5. Molecular pathways leading to apoptosis in mammalian cells. Activation of the extrinsic pathway begins with the binding of soluble or cell surface-expressed ligands to death receptors, such as TNF-R1, FasR and TRAIL, on the plasma membrane. This stimulates formation of the death-inducing signaling complex (DISC) which catalyses the activation of caspase-8. Caspase-8 then directly activates the executioner caspase, caspase-3, which leads to apoptotic cell death. Alternatively, in response to various stress stimuli, the intrinsic pathway is initiated through the activation and translocation of pro-apoptotic Bcl-2 family proteins, such as Bax, to the mitochondrial surface. Genotoxic stress also activates p53 which initiates the transcriptional upregulation of Bax, and the pro-apoptotic BH3-ony proteins Puma and NOXA. Permeabilisation of the OMM by Bax and Bak homo-oligomers releases cytochrome c into the cytosol which is required for the activation of capsae-9 via the apoptosome. Caspase-9 then activates executioner caspases. Cross-talk between these two pathways occurs as a result of caspase-8-mediated cleavage of Bid to tBid which then promotes Bax activation, translocation and/or oligomerisation on the OMM.

proteins and contain a C-terminal intracellular tail, a membrane-spanning region and an extracellular ligand-binding N-terminal domain (Schulze-Osthoff *et al.*, 1998). Respective ligands for the above receptors belong to the TNF superfamily (Schmitz *et al.*, 2000), and are typically found either as transmembrane proteins expressed on the surface of specialised cells, such as Fas ligand (FasL/CD95L) on activated T cells (Berke, 1995), or as soluble molecules (cytokines), such as TRAIL and TNFα released from macrophages, T cells and B cells (Yoon and Gores, 2002).

The cytoplasmic tails of the death receptors, together with a specialised adapter protein known as Fas-associated death domain (FADD) recruit initiator procaspase-8 by means of homologous binding between death effector domains (DEDs) on FADD and within the prodomain of procaspase-8 (Muzio *et al.*, 1996). Together, this forms the death-inducing signalling complex (DISC) which ramps up the autocatalytic activation of procaspase-8 (Boatright *et al.*, 2003). Mature caspase-8 then directly cleaves and activates the effector caspases, -3 and -7, which leads to apoptotic cell death..

The above overview describes the pathway found in what are known as type I cells, typified by peripheral T cells and thymocytes, in which the death receptor-mediated activation of initiator caspases leads directly to the activation of the effector caspases-3 and -7 (Scaffidi *et al.*, 1998). However, in type II cells, such as epithelial cells, insufficient caspase-8 is generated from DISC to sustain this direct approach, and so an amplification step is required (Scaffidi *et al.*, 1998). In the type II pathway, activated caspase-8 cleaves the pro-apoptotic Bcl-2 family protein, Bid (Luo *et al.*, 1998; Li *et al.*, 1998), which initiates the release of cytochrome c from mitochondria. – see Figure 1.5. This biochemical event constitutes a major step within the alternative intrinsic apoptotic pathway, as will be described in the following section. Cytosolic cytochrome c then recruits apoptosis protease activating factor-1 (Apaf-1) and procaspase-9 to form the apoptosome which catalysis the allosteric conformational maturation of this initiator caspase (Rodriguez and Lazebnik, 1999; Zou *et al.*, 1999). Active caspase-9, as with caspase-8, cleaves and activates the effector caspases-3 and -7 to bring about apoptotic

cell death (Li *et al.*, 1997). Thus, in type I cells apoptosis can be induced independently of mitochondrial involvement, whereas in type II cells cytochrome c released from mitochondria is an essential amplification loop to ensure that sufficient quantities of active caspases are generated for apoptosis to run its course (Schmitz *et al.*, 2000). Indeed, in such type II cells, over-expression of the anti-apoptotic Bcl-2 family proteins, Bcl-2 or Bcl-X_L, which function to protect the mitochondria, effectively inhibit Fas-induced apoptosis (Adams and Cory, 1998). Therefore, the concept that the extrinsic and intrinsic routes to apoptosis are always distinct and separate events is an over-simplification of the converging pathways to apoptosis.

1.7.2. The intrinsic (or mitochondrial) pathway

The intrinsic apoptotic pathway (also known as the stress-induced or mitochondrial pathway) is initiated in response to diverse extracellular stimuli, such as ionising radiation, hypoxia, chemotherapeutic agents or withdrawal of growth cues, such as cytokines, NGF and serum (Adams, 2003). In response to such environmental factors, pro-apoptotic proteins belonging to the Bcl-2 family are activated and target the mitochondria where they liberate the release of cytochrome c and other apoptogenic molecules across the outer mitochondrial membrane (OMM). Once in the cytosol, cytochrome c initiates the activation of caspase enzymes, via the apoptosome, and the execution phase of apoptosis commences.

Since this pathway is initiated in response to cellular stress, it is the principle mechanism by which cells undergo apoptosis during or following traumatic brain injury and stroke, as well as underlying the pathological apoptosis associated with neurodegenerative diseases and myocardial ischaemia (Thompson, 1995; Mattson, 2000). As a result, much effort is being employed to develop new therapeutic approaches that specifically target components within this pathway.

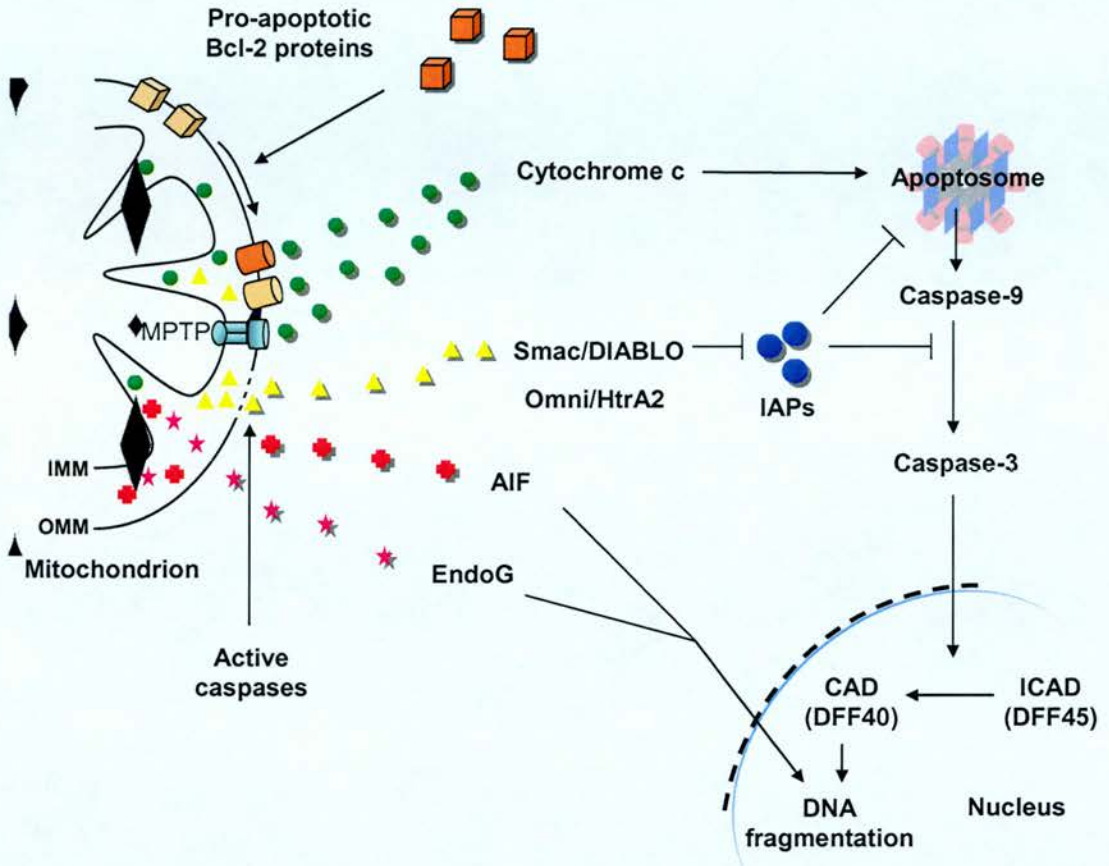

Figure 1.6. Release of apoptogenic factors from the mitochondrial inter-membrane space. The mitochondrial inner membrane space holds a host of apoptogenic factors that are released into the cytosol following permeabilisation of the outer mitochondrial membrane (OMM). Pro-apoptotic Bcl-2 family proteins form multi-meric pores or stimulate opening of the mitochondrial permeability transition pore (MPTP) to release cytochrome c. which is required to form the caspase-9 activating apoptosome complex. The release of smac/DIABLO, which requires active caspases, neutralises inhibitor of apoptosis proteins (IAPs), particularly XIAP, preventing them from suppressing caspase activation. A functionally related protein, the serine protease Omi/HtrA2, also interacts with and neutralises IAPs. The flavoprotein, apoptosis-inducing factor (AIF), and the DNase, endonuclease G (EndoG), translocate into the nucleus where they cleave genomic DNA independently of caspase-activated nucleases, such as CAD/DFF40.

Initiation of the intrinsic pathway is determined by a diverse group of proteins containing both pro- and anti-apoptotic members known as the Bcl-2 protein family, the founding member of which has already been introduced. Bcl-2, together with other anti-apoptotic Bcl-2 family proteins, protects the cell against apoptosis by preventing pro-apoptotic proteins within the same family permeabilising the OMM, thus ensuring containment of the host of apoptogenic molecules that reside within the inter-membrane space (Yang *et al.*, 1997; Kluck *et al.*, 1997). The intrinsic pathway is therefore commonly delineated into events occurring upstream of OMM permeabilisation, which concern the Bcl-2 protein family, and those that follow the release of death-promoting factors from mitochondria into the cytosol leading to the activation of the caspases. However, this model is complicated by other findings which show that caspases can be activated upstream or independently of mitochondrial events (Strasser *et al.*, 2000; Adams, 2003); that the release of certain apoptogenic molecules (for example, smac/DIABLO) is caspase-dependent (Adrain *et al.*, 2001); and that permeabilisation of the OMM can lead to apoptotic cell death independently of caspase activity via the mitochondrial release of such death agents as apoptosis-inducing factor (AIF) (Susin *et al.*, 1999) and endonuclease G (EndoG) (Li *et al.*, 2001) that translocate directly into the nucleus – see Figure 1.6.

1.8. The role of mitochondria in the intrinsic apoptotic pathway

The role of mitochondria as the metabolic engine of the cell, generating more than 90% of available adenosine triphosphate (ATP) by oxidative phosphorylation, and thus essential for life, is well established (Nieminen, 2003). However, with the discovery that Bcl-2 exerts its protective effect at the mitochondrial membrane (Hockenbery *et al.*, 1990) and that a mitochondria-enriched membrane fraction was required for apoptosis to take place in *Xenopus laevis* oocyte extracts (Newmeyer *et al.*, 1994), it became apparent that mitochondria were also a central component of cell death pathways. Moreover, the discovery that the release of the electron carrier, cytochrome c, from the inter mitochondrial membrane space into the cytosol is required for caspase activation

and apoptosis in response to several stress-inducing stimuli, led to the hypothesis that the permeabilisation of the OMM is an essential checkpoint within the intrinsic apoptotic pathway (Liu *et al.*, 1996; Kluck *et al.*, 1997; Li *et al.*, 2000). However, the loss of cytochrome c from the mitochondria has obvious implication for energy metabolism and thus for sustaining the apoptotic process due to its reliance on ATP production (Leist *et al.*, 1997). During Fas-induced apoptosis in mouse hepatocytes, mitochondrial respiration was seen to diminish over time, concomitant with cytochrome c release (Mootha *et al.*, 2001). It has been shown that the soluble fraction of cytochrome c within the inter-membrane space is released early in apoptosis, before the electrochemical gradient ($\Delta\psi m$) across the inner mitochondrial membrane (IMM) driving ATP synthesis has been significantly disrupted (Wei *et al.*, 2000; Shimizu and Tsujimoto, 2000). This initial release phase is thought to be sufficient to induce formation of the apoptosome while the remaining pool of cytochrome c that is more tightly associated with the IMM ensures that the electron transport chain continues operating (van Loo *et al.*, 2002; Ott *et al.*, 2002; Scorrano *et al.*, 2002). Eventually, as cytochrome c continues to be released from mitochondria and apoptosis enters its final stages, $\Delta\psi m$ collapses and mitochondrial respiratory function is no longer sustainable (Mootha *et al.*, 2001; van Loo *et al.*, 2002).

1.9. The Bcl-2 protein family

Proteins belonging to the Bcl-2 family are characterised structurally by their Bcl-2 homology (BH) domains and can be classified into three functionally-distinct subfamilies according to the number of BH domains they posses – see Figure 1.7. Bcl-2 and its four anti-apoptotic relatives, Bcl-X_L, Bcl-w, Mcl-1 and A1, contain all four BH domains (BH 1-4) and protect the mitochondria by sequestering and inhibiting pro-apoptotic Bcl-2 family proteins under normal conditions (Cheng *et al.*, 2001; Cory and Adams, 2002). The pro- apoptotic members of the Bcl-2 protein family can be further sub-divided into two structurally and functionally distinct subgroups, both of which are required for killing (Zong *et al.*, 2001; Cory and Adams, 2002): 1) the multi-BH domain

Sub-family	Bcl-2 family protein	Cellular localisation	Noteable Functions
Anti-apoptotic			
	Bcl-2 (B cell lymphoma-associated protein 2)	OMM, ER & nucelar membranes	Bcl-2, and other multi-domain anti-apoptotic proteins, protect the cell against instrinsic stress stimuli.[1]
	Bcl-w	Loosely attached to OMM	Essential for spermatogeneisis.[2, 3]
	Bcl-X_L (B cell lymphoma protein X Long isoform)	OMM & nuclear membranes	A key regulator of Bax during neuronal development.[4]
	Bcl-X_{ES} (B cell lymphoma protein X extra short isoform)	Cytosol/OMM	Inhibits Bax oligomer formation and Apaf-1 function.[5]
	Mcl-1 (Myeloid-cell leukemia sequence-1)	Cytosol/OMM	Inhibits the pro-apoptotic activity of Bok, Bax and Bak.[6]
	A1	OMM	Required for protection of neutrophils against apoptotic insult.[7]
Pro-apoptotic Multi-domain			
	Bax (Bcl-2 associated protein X)	Cytosol	Permeabilises the OMM to release cytochrome c.[8, 9]
	Bak (Bcl-2 antagonist killer protein)	OMM	Permaebilises the OMM to release cytochrome c.[10]
	Bok (Bcl-2-related ovarian killer protein)	OMM	Important pro-apoptotic protein in reproductive tissues.[11]
	Bcl-X_S (B cell lymphoma protein X short isoform)		A pro-apoptotic cleavage product generted from Bcl-X_L as a result of calpain-mediated proteolysis.[12]
Pro-apoptotic BH3-only proteins			
Death agonists	**Bid** (BH3-interacting-domain death agonist)	Cytosol (sequestered to cytoskeletal proteins)	Truncated by caspase-8 to form active tBid.[13, 14] Promotes Bax and Bak activity.[15, 16]
	Bim (Bcl-2-interacting mediato of cell death; Bim-EL, Bim-L, Bim-s)	Cytosol	Promotes Bax activity.[17] A major apoptogenic protein in the immune system[18] and nervous system.[19]
	Puma (P53 upregulated modulator of apoptosis)	Nucleus/cytosol	Upregulated by p53,[20] promotes Bax activity[21] and inhibts Bcl-2.[22]
Survival antagonists	**Bad** (Bcl-2 antagonist of cell death)	Cytosol (sequestered to 14-3-3 proteins)	Inhibits Bcl-X_L.[23]
	Bik (Bcl-2-interacting killer)	Cytosol	Inhibits Bcl-2 and Bcl-X_L.[24] Required for apoptosis in B cells.[25]
	Bmf (Bcl-2-modifying factor)	Cytosol (sequestered to cytoskeletal proteins)	May be critical to apoptosis induced by anoikis.[26]
	BNIP3	Cytosol	Contributes to hypoxia-acidosis induced apoptosis in myocardial ischemia.[27]
	Hrk (Harakiri)	Cytosol	Implicated in growth-factor-withdrawal-induced apoptosis in certain neuronal cell populations.[28]
	Noxa ("damage-causing")	Cytosol	Upregulated by p53.[29] and inhibits Mcl-1 and A1.[30]

Table 1.2. The Bcl-2 protein family. References: **1, 11**. Cory & Adams, 2002. **2**. Print *et al.*, 1998. **3**. Ross *et al.*, 1998. **4**. Shindler *et al.*, 1997. **5**. Schmitt *et al.*, 2004. **6**. Hsu *et al.*, 1997. **7**. Hamasaki *et al.*, 1998. **8**. Eskes *et al.*, 1998. **9**. Jurgensmeier *et al.*, 1998. **10, 15**. Korsemeyer *et al.*, 2000. **12**. Nakagawa & Yuan, 2000. **13**. Luo *et al.*, 1998. **14**. Li *et al.*, 1998. **16**. Wei *et al.*, 2000. **17**. Marani *et al.*, 2002. **18**. Bouillet *et al.*, 2002. **19**. Putcha *et al.*, 2002. **20, 22**. Nakagawa & Vousden, 2001. **21**. Jeffers *et al.*, 2003. **23**. Schinzel *et al.*, 2004. **24**. Letai *et al.*, 2002. **25**. Jiang & Clark, 2001. **26**. Puthalakath *et al.*, 2001. **27**. Kubasiak *et al.*, 2002. **28**. Imaizumi *et al.*, 2004. **29**. Oda *et al.*, 2000. **30**. Yakovlev *et al.*, 2004.

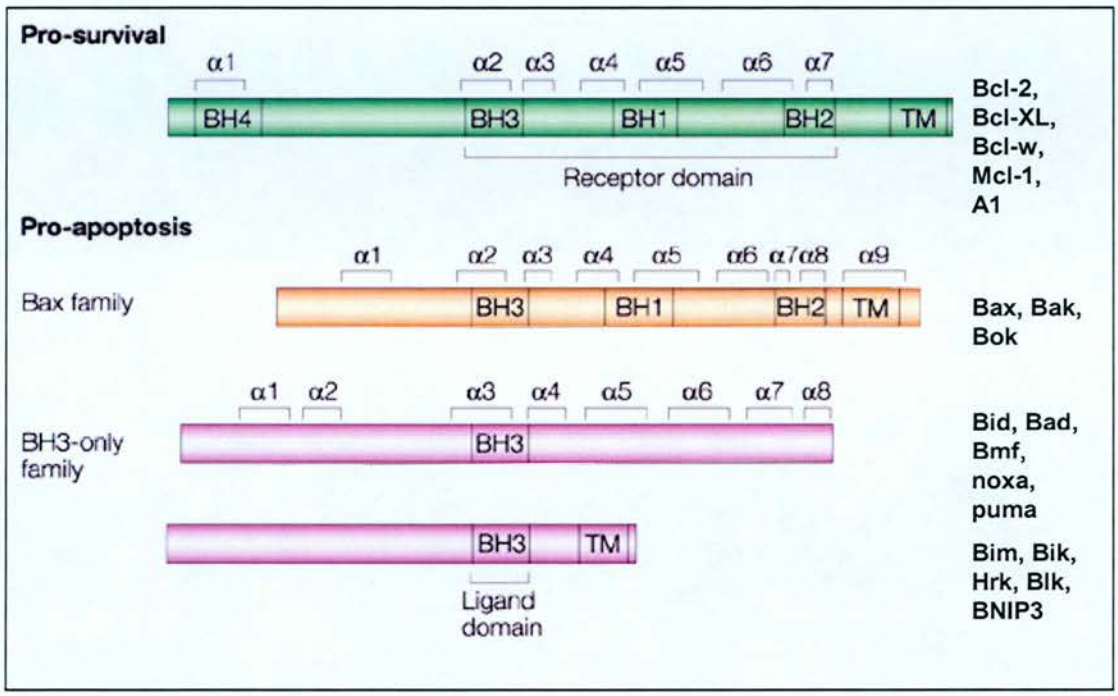

Figure 1.7. Classification of the Bcl-2 protein family according to structure. The Bcl-2 family proteins contain conserved Bcl-2 homology domains (BH domains) in reference to the four BH domains (1-4) initially identified within Bcl-2 and also found within the other multi-domain anti-apoptotic members. All Bcl-2 family proteins contain a BH3 domain which mediates heterodimerisation between opposing members of the family through insertion into a hydrophobic groove (or "receptor") formed by BH domains 1-3 on multidomain proteins. The pro-apoptotic proteins are divided into two sub-families: the multi-domain (BH1-3) proteins, Bax, Bak and Bok, and the numerous BH3-only proteins. A carboxyl-terminal hydrophobic domain that facilitates association with intracellular membranes (Transmembrane (TM) domain) is found in all members except A1 and several BH3-only proteins. Known alpha helices are also depicted. Image adapted from Cory & Adams, 2002.

Bax family, containing Bax and Bak, which are the main effectors of OMM permeabilisation (Wei *et al.*, 2001), as well as Bok, a poorly characterised protein expressed in reproductive tissues (Hsu *et al.*, 1997a), and 2) the numerous BH3-only proteins that function as pro-death signalling factors as well as antagonists to the protective BH1-4 proteins (Cory and Adams, 2002) – see Table 1.2. for a full list and description of these proteins.

1.9.1. Cellular localisation of Bcl-2 family proteins

The principle cellular target of the Bcl-2 protein family is the mitochondria. On the cytosolic face of the OMM reside the anti-apoptotic proteins, Bcl-2, Bcl-X_L, Bcl-w and Mcl-1 as-well as the pro-apoptotic protein, Bak (Schinzel *et al.*, 2004a). In contrast, Bax and the pro-apoptotic BH3-only proteins are predominantly found within the cytosol and therefore must translocate to the OMM during apoptosis in order to make a functional contribution (Sattler *et al.*, 1997; Wolter *et al.*, 1997). The presence of a C-terminal transmembrane domain in many Bcl-2 family proteins determines their cellular localisation to the membrane surfaces of organelles (Nguyen *et al.*, 1993; Kaufmann *et al.*, 2003) and has been implicated as a membrane-targeting domain directing pro-apoptotic proteins through the cytosol to the OMM during apoptosis (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000). As will be discussed in more depth later, regulation of the exposure of this domain in the pro-apoptotic protein Bax is a key determinant of its apoptogenic function and, as such, is currently a key focus area for research.

In addition to mitochondria, Bcl-2 family proteins are also found on, or translocate to, the surface of the ER membrane, where they have been proposed to initiate an ER-directed cell death pathway in response to certain intracellular stimuli, such as the accumulation of misfolded proteins or a perturbed Ca^{2+} homeostasis (Nakagawa *et al.*, 2000; Wei *et al.*, 2001; Scorrano *et al.*, 2003). In fact, the majority of endogenous Bcl-2 (about two thirds) is located on the ER membrane (Lithgow *et al.*, 1994; Schinzel *et al.*,

2004a) and the pro-apoptotic proteins, Bax and Bak, have been observed to translocate there (Nutt *et al.*, 2002; Scorrano *et al.*, 2003).

1.9.2. Structure-function relationships within the Bcl-2 protein family

For nearly a decade following the discovery of Bcl-2, the underlying mechanism by which the Bcl-2 family of proteins regulated apoptosis remained unknown (Korsmeyer, 1995). However, detailed structural analyses of Bcl-2 family proteins, together with genetic studies in *C. elegans*, finally provided an insight into how these proteins were able to regulate their own activity and thus determine the fate of the cell in response to stress signals. The first breakthrough came with the elucidation of the structure of the anti-apoptotic Bcl-2 family member, Bcl-X_L, using X-ray crystallography and nuclear magnetic resonance (NMR) (Muchmore *et al.*, 1996). This study revealed Bcl-X_L to have a central hydrophobic core consisting of two alpha helices surrounded by five amphipathic α helices to form a structure that bore close resemblance to certain pore-forming bacterial toxins, namely diphtheria toxin and the colicins (Parker and Pattus, 1993). This observation gave rise to the theory that pro-apoptotic members belonging to the Bcl-2 protein family could form membrane pores or ion channels in order to liberate apoptogenic molecules across intracellular membranes, a mechanism that still has support today but is currently difficult to prove in a real-time *in vivo* setting (Sharpe *et al.*, 2004). The crystal structure of Bcl-X_L also illustrated how the orientation of the BH domains formed a hydrophobic groove that was proposed to form a putative binding site for other Bcl-2 family proteins (Muchmore *et al.*, 1996). The following year it was demonstrated that the amphipathic helical BH3 domain belonging to one Bcl-2 family member could insert into the hydrophobic pocket of another (Sattler *et al.*, 1997) – see Figure 1.8. This had important implications for explaining how opposing members of the Bcl-2 protein family, for example the BH1-4 anti-apoptotic proteins and the pro-apoptotic BH3-only proteins, could potentially interact and neutralise each other. The theory that interaction between opposing members of the Bcl-2 protein family controlled their activity was first put forward by researchers working in the laboratory of Stanley

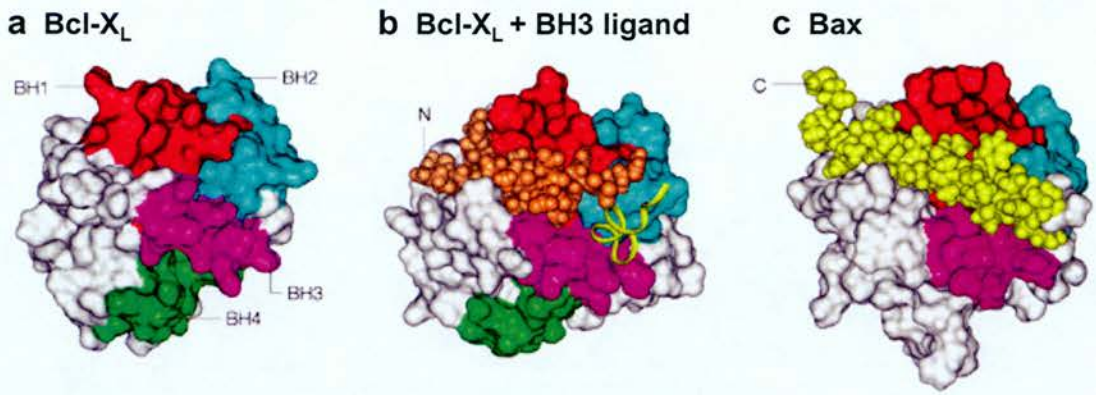

Figure 1.8. Three-dimensional structures of Bcl-xL and Bax, showing their similarity. **(a)** Bcl-xL, with its four BH domains labelled. **(b)** Bcl-xL with the BH3 peptide of Bak (brown) bound to its surface groove. **(c)** Bax, showing its C-terminal tail (yellow) tucked into the groove, but running in the opposite orientation to a BH3 ligand. Image taken from Cory & Adams, 2002.

Korsmeyer who discovered the pro-apoptotic Bcl-2 family member, Bax, as a binding partner of Bcl-2 (Oltvai *et al.*, 1993). Further proof that heterodimerisation amongst Bcl-2 family proteins was a central regulatory mechanism arose from genetic studies in *C. elegans* where the BH3-only protein homolog, EGL-1, directly interacted with and suppressed the anti-apoptotic Bcl-2 homolog, CED-9 (Conradt and Horvitz, 1998). The mechanism by which opposing Bcl-2 family proteins heterodimerise was also proposed to mediate homodimerisation between multi-domain pro-apoptotic Bcl-2 family proteins, such as Bax and Bak, into large proteinaceous channels across the OMM (Chittenden *et al.*, 1995; Zha *et al.*, 1996; Simonian *et al.*, 1996). However, despite these early reports supporting the requirement of the Bax/Bak BH3 domain for homo-oligomerisation, subsequent studies have failed to establish their validity and so the domains within Bax and Bak responsible for their oligomerisation on the OMM remain unknown (Sharpe *et al.*, 2004). Nevertheless, as will be discussed in more detail later, the ability of Bax and Bak to from such homo-oligomers is thought to be crucial to their pro-apoptotic activity (Gross *et al.*, 1998; Wei *et al.*, 2000; Antonsson *et al.*, 2000; Korsmeyer *et al.*, 2000; Nechushtan *et al.*, 2001) as it provides a possible mechanism by which they are able to destabilise the OMM or assemble themselves into trans-membrane pores of sufficient size to mediate the release of death-promoting proteins across the OMM.

1.9.3. The pro-apoptotic BH3-only proteins

Under normal conditions, the majority of pro-apoptotic BH3-only proteins lie dormant and dispersed throughout the cytosol (Sattler *et al.*, 1997). However, upon induction of apoptosis, these proteins are either transcriptionally upregulated or liberated from various post-translational checkpoints - see Table 1.3. It is therefore currently thought that the BH3-only proteins represent the early responders within the Bcl-2 family to incoming stress signals or developmental cues (Adams, 2003). Once liberated, BH3-only proteins target either multi-domain anti-apoptotic proteins, such as Bcl-2, Bcl-$_{XL}$, Mcl-1 and Bcl-w on the OMM, using their BH3 domains to displace sequestered pro-

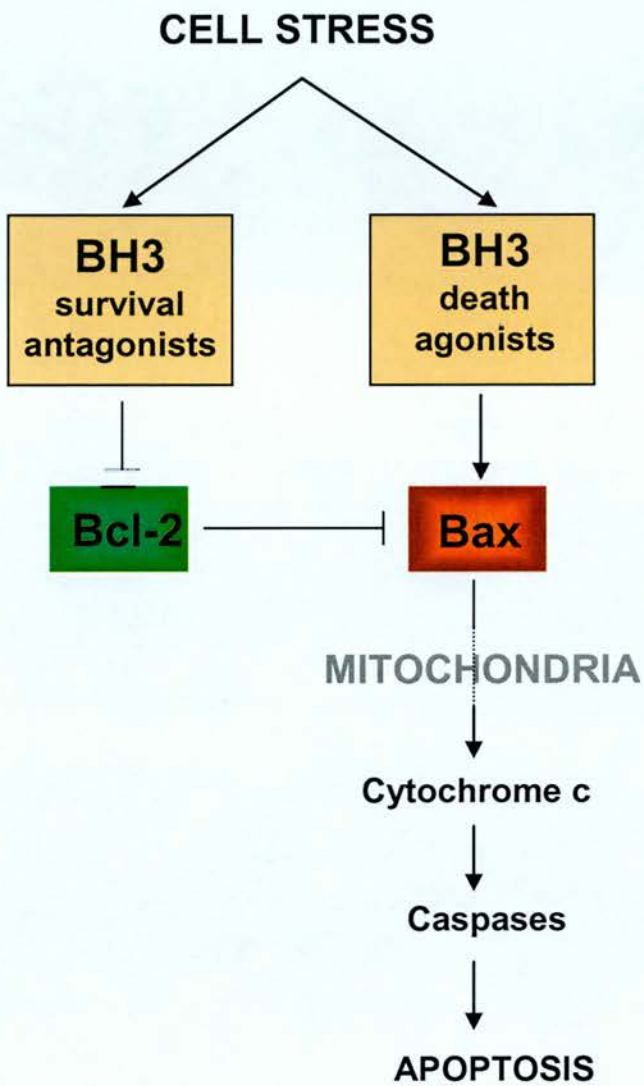

Figure 1.9. Differential targeting by BH3-only proteins. During stress-induced apoptosis, BH3-only "survival agonists" translocate to the OMM and interact with anti-apoptotic Bcl-2 proteins in order to neutralise their activity. This targeting is mediated either through having high affinity for multi-domain Bcl-2 family members, or by"death agonists" utilising C-terminal TM domains, as is the case for Bik, Hrk, Blk, and Bim. Alternatively, the BH3-only proteins Bid/tBid, Bim-s and PUMA, are known to also interact directly with pro-apoptotic multi-domain Bcl-2 family proteins, such as Bax, to stimulate their activity.

Regulatory control	BH3-only protein	Stimulus	References
Transcriptional	NOXA	DNA damage	1
	PUMA	DNA damage	2
	Bim_S	Growth factor deprivation	3
	Hrk	Growth factor deprivation	4
Post-translational modification:			
Cleavage	Bid	Death receptor ligation	5, 6
Dephosporylation	Bad	Growth factor deprivation/anoikis	7
Phosphorylation	Bik	?	8
Dissocation from:			
14-3-3 scaffold proteins	Bad	Growth factor deprivation	9
Dynein light chain 1	Bim_{EL}, Bim_L	Ca^{2+} flux/taxol	10
Dynein light chain 2	Bmf	UV irradiation/anoikis	11

Table 1.3. Regulation of BH3-only protein activation. BH3-only proteins, as sentinels for cell stress within the Bcl-2 protein family, respond to diverse stimuli and require either transcriptional or post-translation-modifications to become active death-promoting proteins. References: **1.** Oda *et al.*, 2000. **2.** Nakano & Vousden, 2001. **3.** Putcha *et al.*, 2001. **4.** Imaizumi *et al.*, 1999. **5.** Luo *et al.*, 1998. **6.** Li *et al.*, 1998. **7.** Wang *et al.*, 1999. **8.** Verma *et al.*, 2001. **9.** Zha *et al.*, 1996. **10.** Puthalakath *et al.*, 1999. **11.** Puthalakath *et al.*, 2001.

apoptotic proteins (Yang *et al.*, 1995; Wilson-Annan *et al.*, 2003; Willis *et al.*, 2005), or promote the activation of the effector pro-apoptotic proteins of the Bcl-2 family, Bax and Bak (Wang *et al.*, 1996; Desagher *et al.*, 1999; Cartron *et al.*, 2004; Kuwana *et al.*, 2005) – see Figure 1.9.

1.9.4. The multi-domain pro-apoptotic proteins

The multi-domain proteins, Bax and Bak, share extensive structural homology and together represent the core components of the pro-apoptotic Bcl-2 family protein machinery (Lindsten *et al.*, 2000; Wei *et al.*, 2001). This authoritative position is demonstrated by the many observations that, in the absence of both Bax and Bak, none of the remaining pro-apoptotic Bcl-2 family proteins are able to breach the OMM and liberate the apoptogenic molecules within (Wei *et al.*, 2001; Zong *et al.*, 2001; Cheng *et al.*, 2001). From genetic studies, the inactivation of both genes ($Bax^{-/-}$ $Bak^{-/-}$) was observed to dramatically impair apoptosis in a wide variety of tissues in response to classical activators of the intrinsic pathway, such as ionising radiation, DNA-damaging drugs, the kinase inhibitor staurosporine and growth factor deprivation (Lindsten *et al.*, 2000; Wei *et al.*, 2001; Cheng *et al.*, 2001). Moreover, few $Bax^{-/-}Bak^{-/-}$ mice survive to adulthood and those that do exhibit severe developmental defects in multiple tissues as well as impaired regulation of tissue homeostasis (Lindsten *et al.*, 2000; Lindsten *et al.*, 2003) In contrast, disrupting *Bak* gene expression alone in mice ($Bak^{-/-}$) does not appear to result in any observable phenotypic abnormalities (Lindsten *et al.*, 2000), whereas obvious developmental abnormalities were observed in $Bax^{-/-}$ mice, such as increased populations of sympathetic and facial motor neurones indicating defective developmental neuronal cell death (Deckwerth *et al.*, 1996) and infertility in the males due to impaired spermatogenesis (Knudson *et al.*, 1995). This therefore suggests that the intrinsic apoptotic pathway in certain tissues is Bax-dependent. However, both $Bak^{-/-}$ and $Bax^{-/-}$ knock-out animals survive into adulthood (Knudson *et al.*, 1995; Lindsten *et al.*, 2000) which suggests that a certain degree of functional redundancy exists between these two proteins.

How Bak and Bax bring about the release of apoptogenic molecules from the mitochondrial inter-membrane space has yet to be conclusively determined (Sharpe *et al.*, 2004). One enduring hypothesis, based on the structural resemblance of certain Bcl-2 family members to diphtheria toxin (Muchmore *et al.*, 1996) is that both Bax and Bak form pore-like channels within the OMM through which apoptogenic molecules can pass. This model is perhaps most convincingly supported by the finding that oligomers of Bax can form pores in liposomes that can permit the passage of cytochrome c (Saito *et al.*, 2000; Antonsson *et al.*, 2000) as well as larger molecules (Kuwana *et al.*, 2002). Furthermore, several studies have demonstrated that both proteins insert into the OMM and oligomerise into large complexes during apoptosis to form pore-like structures (Nechushtan *et al.*, 2001; Antonsson *et al.*, 2001). However, although the existence of these pores is well documented, their precise nature and structure remains unresolved (Adams, 2003; Newmeyer and Ferguson-Miller, 2003). There is also evidence consistent with an alternative model in which Bax interacts with and stimulates opening of a large conductance channel spanning the IMM and OMM called the mitochondrial permeability transition (MPT) pore (Tsujimoto and Shimizu, 2000; Zamzami and Kroemer, 2001). In support of this hypothesis, Bax has been shown to be capable of binding to the MPT pore component, voltage-dependent anion channel (VDAC) on the OMM (Narita *et al.*, 1998; Shimizu *et al.*, 1999). Opening of the MTP pore leads to a collapse in $\Delta\psi m$ and the influx of water into the mitochondrial matrix (Green and Reed, 1998). The resultant swelling of the matrix ruptures the OMM and releases soluble molecules within the inter-membrane space into the cytosol (Green and Reed, 1998). A collapse in $\Delta\psi m$, matrix swelling and rupture of the OMM are all features associated with apoptosis (Petit *et al.*, 1996), however it has been shown that none of these events are required for Bax and/or Bak to elicit cytochrome c release and caspase activation (Bossy-Wetzel *et al.*, 1998; Green and Reed, 1998). This therefore suggests that an alternative mechanism of OMM permeabilisation is employed by these proteins.

1.10. Bax

The multi-domain pro-apoptotic protein, Bcl-2 associated protein X (Bax), is a keystone protein within the Bcl-2 family as it translates the intent of the pro-apoptotic BH3-only proteins into direct action at the mitochondrial surface through permeabilisation of the OMM (Cory and Adams, 2002).

Despite containing a C-terminal transmembrane domain, the majority of endogenous Bax (estimates range from 60-80%) is found as a soluble monomer in the cytosol of healthy cells (Wolter *et al.*, 1997; Hsu and Youle, 1998; Nechushtan *et al.*, 2001) with the remainder found loosely attached on the surface of mitochondria and the endoplasmic reticulum (Eskes *et al.*, 2000; Scorrano *et al.*, 2003). Thus, unlike Bak, which resides on the OMM in complex with Mcl-1 and Bcl-X_L (Cuconati *et al.*, 2003; Willis *et al.*, 2005), the majority of endogenous Bax has to first pass through the cytosolic milieu before it can elicit a cytotoxic effect at the mitochondrial surface (Wolter *et al.*, 1997; Hsu *et al.*, 1997b; Goping *et al.*, 1998). This secondary, post-transcriptional level of regulation is thought to ensure that, under normal conditions, a protein as potentially lethal as Bax is maintained in a subcellular location distinct from its site of action (Schinzel *et al.*, 2004a).

How Bax becomes activated remains uncertain (Adams, 2003; Lucken-Ardjomande and Martinou, 2005). One possibility involves the pro-apoptotic BH3-only protein Bid (Desagher *et al.*, 1999; Eskes *et al.*, 2000). In response to death receptor activation, Bid is cleaved into a 15.5kDa fragment containing its BH3 domain and C-terminal tail (tBid) by caspase-8 (Luo *et al.*, 1998; Li *et al.*, 1998). tBid is then myristoylated which leads to its translocation to the OMM (Zha *et al.*, 2000) where it has been proposed to engage Bax and promote its oligomerisation (Desagher *et al.*, 1999; Eskes *et al.*, 2000; Korsmeyer *et al.*, 2000; Roucou *et al.*, 2002; Kuwana *et al.*, 2002). However, this interaction, if it does occur, must be only a transient one as so far co-localisation of tBid and Bax on the mitochondrial surface has not been observed (Nechushtan *et al.*, 2001)

and neither has tBid been identified as a component of Bax oligomers (Wei *et al.*, 2000; Grinberg *et al.*, 2002). Alternatively, tBid may intercept Bax before it has undergone translocation to the OMM in order to initiate this event (Cartron *et al.*, 2004). Supporting evidence for this arose from the recent identification of a novel binding site within the N-terminal α1 helix of Bax that received Bid (and the BH3-only protein PUMA) leading to ligand-induced Bax activation and its subsequent translocation to the OMM (Cartron *et al.*, 2004). However, in the absence of Bid, although Fas-mediated apoptosis in hepatocytes is impaired (Yin *et al.*, 1999) stress-induced apoptosis that relies on OMM permeabilisation is not (Wei *et al.*, 2001), suggesting that other regulatory factors/proteins play a hand in orchestrating Bax activation.

1.10.1. The role of Bax in neuronal apoptosis

Gene deletion studies have demonstrated that Bax makes an important contribution to neuronal apoptotic pathways initiated during development and in response to neuropathology (Deckwerth *et al.*, 1996; White *et al.*, 1998). As has already been discussed, the elimination of synapse-bearing neurones by apoptosis in the developing nervous system is a central mechanism through which neuronal cell numbers in the adult brain are determined (Oppenheim, 1991). This process is largely orchestrated by restricting the availability of target-derived neurotrophic support within the CNS during this developmental phase, which leads to the initiation of the intrinsic apoptotic pathway in neurotrophin-deprived cells (Deckwerth and Johnson, Jr., 1993; Edwards and Tolkovsky, 1994). Consequently, Bax-knockout mice exhibit a markedly reduced apoptotic programme within the developing nervous system, which leads to increased neuronal cell numbers in a variety of sites, including the brainstem, cerebellum, dorsal root ganglia, hippocampus and spinal cord (Deckwerth *et al.*, 1996; White *et al.*, 1998). In contrast, the influence of Bax deletion in the development of non-neuronal tissues appears to be less noticeable (Knudson *et al.*, 1995). This observed dependence of neuronal tissue on Bax function is also seen *in vitro*, where the withdrawal of NGF from cultured sympathetic neurons lacking Bax does not initiate cytochrome c release and

apoptotic death, as is observed in wild type cultures (Deckwerth *et al.*, 1996; Deshmukh and Johnson, Jr., 1998).

Increased Bax activity has also been associated with certain types of neurodegeneration, particularly seizure-induced neurodegeneration (diaschisis) (Zhang *et al.*, 1998; Tuunanen *et al.*, 1999) and neurodegeneration resulting from ethanol-induced neonatal brain injury (Young *et al.*, 2003) and nerve injury, for example that following axotomy of motoneurones (Gillardon *et al.*, 1996a) or the optic nerve (Isenmann *et al.*, 1997). Increased expression of Bax has also been identified in several chronic neurodegenerative diseases, including ALS and Alzheimer's disease. Increased expression of Bax was identified in transgenic mouse models of ALS (Vukosavic *et al.*, 1999) as well as in the motor cortex and spinal cord motor neurons of patients who had died from ALS (Mu *et al.*, 1996; Ekegren *et al.*, 1999), and the upregulation of several pro-apoptotic proteins, including Bax, has been observed in neurons associated with amyloid deposits in the brains of Alzheimer's patients (Tortosa *et al.*, 1998; Mattson, 2000). In animal models of global cerebral ischeamia, increased Bax expression has been observed in neurones within ischemia-sensitive regions of the brain, such as the CA1 sector of the hippocampus (Krajewski *et al.*, 1995; Hara *et al.*, 1996), and, in a rodent model of middle cerebral artery occlusion, elevated Bax protein expression was identified in the penumbral region of focal infarcts (Gillardon *et al.*, 1996b). Targeting the apoptogenic activity of Bax by therapeutic intervention could therefore reduce the extent of neuronal apoptotic cell death observed in all of these disorders.

1.10.2. Bax structure

Insights into how the mitochondrial targeting of Bax is regulated came from the partial elucidation of the soluble Bax structure by NMR technology (Suzuki *et al.*, 2000). This study revealed that the Bax protein structure bears many similarities to other multi-domain proteins within the Bcl-2 protein family, such Bcl-X_L, the structure of which had also been determined (Muchmore *et al.*, 1996). However, unlike the anti-apoptotic

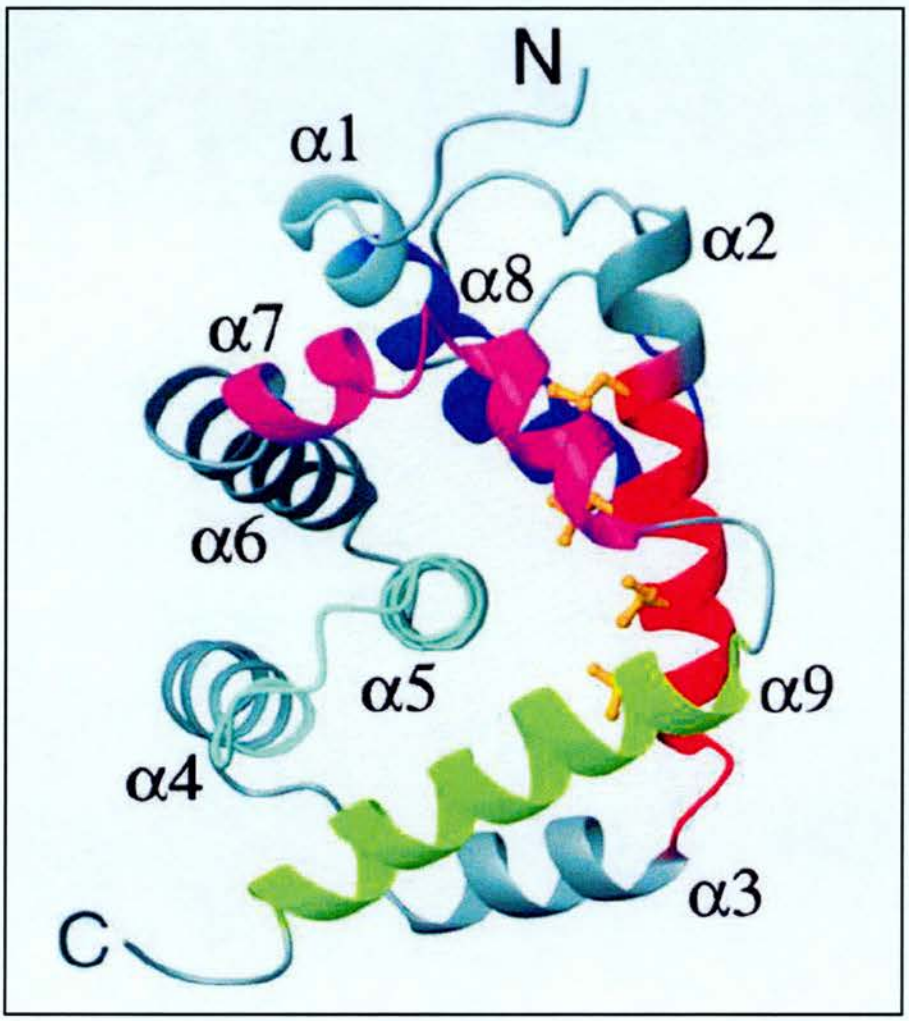

Figure 1.10. Structure of Bax. A ribbon representation of the NMR structure of Bax, viewed straight down the central hydrophobic α5 helix. The BH1-3 domains are shown in cyan, magenta and red, respectively. The C-terminal helix (α9) is shown in green. Image taken from Suzuki et al, 2000.

multidomain proteins, Bax does not contain a fourth BH domain (Suzuki *et al.*, 2000). Eight amphipathic α helices within Bax are arranged around a central hydrophobic α helix (α5), forming a hydrophobic core to the protein – see Figure 1.10. The BH3 domain, which is required for the activity of pro-apoptotic members in the Bcl-2 family, is in α helix 2. BH domain 1, 2 and 3 enclose a hydrophobic pocket into which the BH3 domain from a partner protein (eg, another Bax protein or Bid/tBid) has been proposed to insert (Sattler *et al.*, 1997). Significantly, the α9 helix, which forms part of the C-terminal transmembrane domain, normally occupies this region, preventing accessibility to the BH3 domains of other proteins under resting cellular conditions (Suzuki *et al.*, 2000). The intra-molecular folding of the transmembrane domain, which is highly hydrophobic, increases the solubility of Bax, thus allowing it to reside as a soluble entity within the cytosol (Suzuki *et al.*, 2000; Schinzel *et al.*, 2004a). This arrangement, whereby the transmembrane domain is folded back into a hydrophobic pocket, is also found in the anti-apoptotic Bcl-2 family protein, Bcl-w, which resides loosely attached to the OMM under normal conditions (Hinds *et al.*, 2003; Denisov *et al.*, 2003; Adams, 2003). However, during apoptosis, displacement of the Bcl-w C-terminal transmembrane domain out of the hydrophobic pocket by a BH3-only protein leads to the insertion of Bcl-w into the OMM, in a process presumably directed by the now exposed transmembrane domain (Wilson-Annan *et al.*, 2003). Similarly, the C-terminal transmembrane domain of Bax, when exposed during apoptosis, is implicated in directing its translocation to the mitochondrial surface and mediating, either solely or in collaboration with other domains, insertion into the OMM (Wolter *et al.*, 1997; Goping *et al.*, 1998; Eskes *et al.*, 2000; Annis *et al.*, 2005). However, in order to accommodate a BH3 domain within the hydrophobic pocket, structural analysis has predicted that Bax has to undergo significant structural alterations involving not just the "unhinging" of the C-terminal α9 helix but also other, distinct regions of the molecule, such as the N-terminus (Goping *et al.*, 1998; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000). Structural studies by Suzuki and colleagues determined that the Bax N-terminus is flexible and solvent-exposed; however, it is a well documented phenomenon that certain residues with the N-terminus (Pro13-Ile19; known as the 6A7 epitope; Hsu & Youle, 1997)

remain hidden in cytosolic Bax and are only exposed and thus available to antibody binding during apoptosis (Hsu and Youle, 1997; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000; Cartron *et al.*, 2002). Furthermore, removal of the first 20 residues of Bax, a region known as the apoptosis regulation of translocation (ART) domain, results in increased targeting of Bax to mitochondria through enforced exposure of the C-terminal transmembrane domain (Goping *et al.*, 1998; Ruffolo *et al.*, 2000) – see Figure 1.11 for an overview of the key regulatory domains within Bax. These observations led to the proposal that the N-terminus of Bax exerts an inhibitory effect on the exposure of the C-terminal transmembrane domain through direct interaction when Bax is cytosolic (Goping *et al.*, 1998; Nechushtan *et al.*, 1999; Arokium *et al.*, 2004; Schinzel *et al.*, 2004b). Recent work has illuminated the role of a proline residue found within a solvent exposed loop region directly preceding the C-terminal transmembrane domain (Pro168) that has been proposed to link the C-and N-termini of Bax (Schinzel *et al.*, 2004b). This bridging residue may therefore establish an allosteric mechanism through which structural alterations occurring in one terminal domain can directly influence those occurring in the other. Thus one possible model of how Bax becomes activated is that, in response to an appropriate apoptotic stimulus, N-terminal conformational alteration, perhaps triggered following interaction with tBid or PUMA, regulates the unhinging of the C-terminal transmembrane domain which in turn directs the targeting of Bax to the OMM (Sharpe *et al.*, 2004; Schinzel *et al.*, 2004a; Lucken-Ardjomande and Martinou, 2005).

Following on from the observation made by Muchmore and colleagues that the arrangement of α helices 5 and 6 in Bcl-X_L resembled that of certain bacterial pore-forming toxins (Muchmore *et al.*, 1996), structural comparisons with other Bcl-2 family proteins also established this region as a putative pore-forming domain in the pro-apoptotic proteins Bid and Bax (Schendel *et al.*, 1998; Chou *et al.*, 1999; McDonnell *et al.*, 1999). These findings provided one possible explanation for the ability of Bax and other Bcl-2 family proteins to form ion-conducting pores in synthetic lipid membranes (Antonsson *et al.*, 1997; Schendel *et al.*, 1998; Matsuyama *et al.*, 1998). Based on the

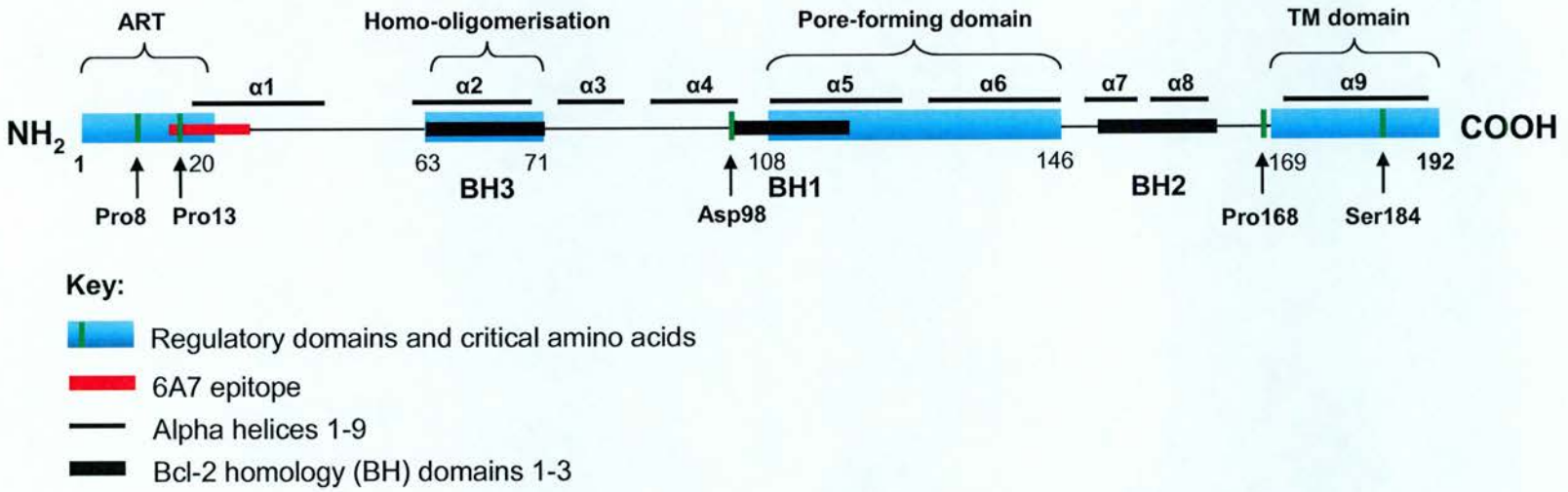

Figure 1.11. Critical amino acids and regulatory domains involved in the bioactivity of Bax. The first 20 amino acids, encompassing the apoptosis regulation of targeting (ART) domain, are believed to regulate exposure of the Bax C-terminal transmembrane (TM) domain. Residues Proline(Pro)8 and Pro13 within this region are thought to be particularly crucial as their specific mutation leads to conformational change, translocation and oligomerisation of Bax. The 6A7-reactive epitope (Pro12-ILe24) is solvent exposed only in conformationally-activated Bax. The BH3 domain mediates homo-oligomerisation to form pore-like structures on the OMM. The central amphipathic α5 and α6 helices form the putative pore-forming domain, which has been proposed to mediate cytochrome c release across the OMM. The final 23 amino acids, which includes the α9 helix, represents the TM domain. Serine (Ser)184 regulates the orientation of this domain via hydrogen bond formation with Aspergine (Asp)98 in the α4 helix. Pro168, located within a loop preceding the TM domain, has been proposed to regulate Bax conformational change and targeting by forming an interface between the N-terminal ART domain and the C-terminal TM domain.

mechanism used by the structurally related colicin bactericidal toxins, which form pores using a hairpin pair of α helices (Cramer *et al.*, 1995), the polar residues of the amphipathic α helices 5 and 6 of Bax, when inserted into the OMM, were proposed to form the aqueous surface of the channel (Nouraini *et al.*, 2000). Two recent studies suggest that the pore-forming domain is a critical component of the strategy used by Bax to permeabilise the OMM (Heimlich *et al.*, 2004; Annis *et al.*, 2005). Bax mutants lacking the pore forming domain, but not those lacking the transmembrane domain or having an impaired BH3 domain, were unable to elicit cytochrome c release from isolated mitochondria, suggesting that neither the transmembrane nor BH3 domains are required to permeabilise the OMM (Heimlich *et al.*, 2004). However, although both studies, together with another recent report (Cartron *et al.*, 2005), agree that the pore-forming domain is essential for Bax insertion into the OMM, Annis and colleagues proposed that this process was the result of a concerted effort between the C-terminal α helix 9 and the α helices 5 and 6. This study also suggested that the subsequent oligomerisation of multiple Bax proteins is required to form pores of sufficient size to release apoptogenic molecules across the OMM (Annis *et al.*, 2005).

1.10.3. Regulation of Bax translocation

Both the C- and N-termini of Bax have been implicated in directing mitochondrial targeting during apoptosis (Wolter *et al.*, 1997; Goping *et al.*, 1998; Cartron *et al.*, 2003; Schinzel *et al.*, 2004b) and the assignment of specific roles to each of these regions remains contentious. However, several separate studies agree that the C-terminal transmembrane domain makes an essential contribution to this process, as the mutation or deletion of all or part of this region prevents Bax from reaching the OMM during apoptosis (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999; Schinzel *et al.*, 2004b). Furthermore, some Bax mutants, in which specific residues within the C-terminal tail are modified, instead exhibit constitutive mitochondrial targeting and heightened toxicity as a consequence of prematurely exposing this region to the cytosol (Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000). One amino acid in particular, Serine 184 (S184), appears to

be critical in regulating the position of the C-terminal tail within the quiescent Bax molecule. S184, located within helix α9, is the only polar residue in this otherwise hydrophobic region to face inwards towards the protein core instead of contributing to its solvent-exposed face (Suzuki *et al.*, 2000). The interactions between the internalised transmembrane domain (of which helix α9 forms a part) and the BH3-binding pocket are all hydrophobic, apart, that is, from a single hydrogen bond between S184 and an asparagine (A98) in helix α4 (Suzuki *et al.*, 2000). Dissipating this bond, either by deleting S184 (ΔS184) or replacing it with a non-polar residue, such as a valine (S184V) or alanine (S184A), triggers the exposure of the transmembrane domain to the cytosol - an event normally only associated with apoptotic stimuli. Consequently, Bax-ΔS184, S184V or S184A mutants are rapidly targeted to the OMM, even in healthy cells, and exhibit high cytotoxicity (Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000).

A comparable outcome is seen when, instead of using full length Bax mutants as above, these experiments are repeated using peptides corresponding to the final 21aa of Bax (Trp170-Gly192) which encompasses the α9 helix (Trp170-Pro188) (Nechushtan *et al.*, 1999). GFP fused to a wild-type Bax C-terminal peptide remained cytosolic when introduced into cells, even in the presence of an apoptotic stimulus (Nechushtan *et al.*, 1999). However, when this experiment was repeated using a peptide containing a ΔS184, S184V or S184A mutation, GFP was instead targeted directly to mitochondria and remained there irrespective of whether an apoptotic stimulus was present or not (Nechushtan *et al.*, 1999). From this observation, S184 appears to not only regulate the unhinging of the C-terminal tail, but somehow also contributes to making the transmembrane domain competent for mitochondrial targeting. When this experiment was repeated by Schinzel and colleagues several years later, in addition to the same 21aa Bax C-terminal peptides, they also introduced into cells peptides corresponding to the final 22 amino acids (Thr169-Gly192) and 23 amino acids (Pro168-Gly192) of the Bax C-terminus (Schinzel *et al.*, 2004b). In agreement with Nechushtan and colleagues, the wild type 21aa peptide remained cytosolic in both healthy and apoptotic cells (Nechushtan *et al.*, 1999), as did the 22aa peptide. However, when they introduced GFP

fused to the 23aa peptide, which includes residues within the solvent exposed loop region directly preceding the α9 helix, they observed that it specifically co-localised with mitochondrial markers as effectively as the mutant S184V peptide used previously (Schinzel *et al.*, 2004b). This important finding therefore suggests that the Bax C-terminal transmembrane domain alone is in fact all that is required to target the whole Bax protein to the OMM.

Thus, the activation, mitochondrial targeting and insertion of Bax into the OMM are controlled by several distinct domains that become functionally active as a result of extensive remodelling of the Bax protein structure. The transformation of Bax into an actively apoptogenic protein is thought to be aided, in part, by the BH3-only protein Bid/tBid (along with, perhaps, other BH3-only proteins, such as Bim-s and PUMA) and suppressed by the anti-apoptotic Bcl-2 family proteins, as has already been described. However, a large body of current and historical data suggest that the influence of each of these regulatory Bcl-2 sub-families on Bax is largely confined to the mitochondrial surface and only take effect once Bax has already undergone its initial activation and translocation steps (Oltvai *et al.*, 1993; Sedlak *et al.*, 1995; Desagher *et al.*, 1999; Eskes *et al.*, 2000; Korsmeyer *et al.*, 2000; Roucou *et al.*, 2002; Kuwana *et al.*, 2002; Terrones *et al.*, 2004). Therefore additional proteins that may regulate earlier steps within the Bax activation pathway, such as its conformational alteration or translocation steps – see Figure 1.12 – have been searched for and several endogenous Bax agonists and antagonists have been recently identified (reviewed by Lucken-Ardjomande & Martinou, 2005). However, when this thesis commenced early in 2004, few endogenous Bax-binding proteins had been discovered. This therefore left two important questions pertinent to the bioactivity of Bax unanswered: 1) is Bax sequestered in the cytosol of quiescent cells through association with retention factors, and 2) does Bax rely on chaperones during its translocation to the OMM. These are the questions that I have addressed within this thesis, the results of which are presented in chapters 4 and 6, respectively.

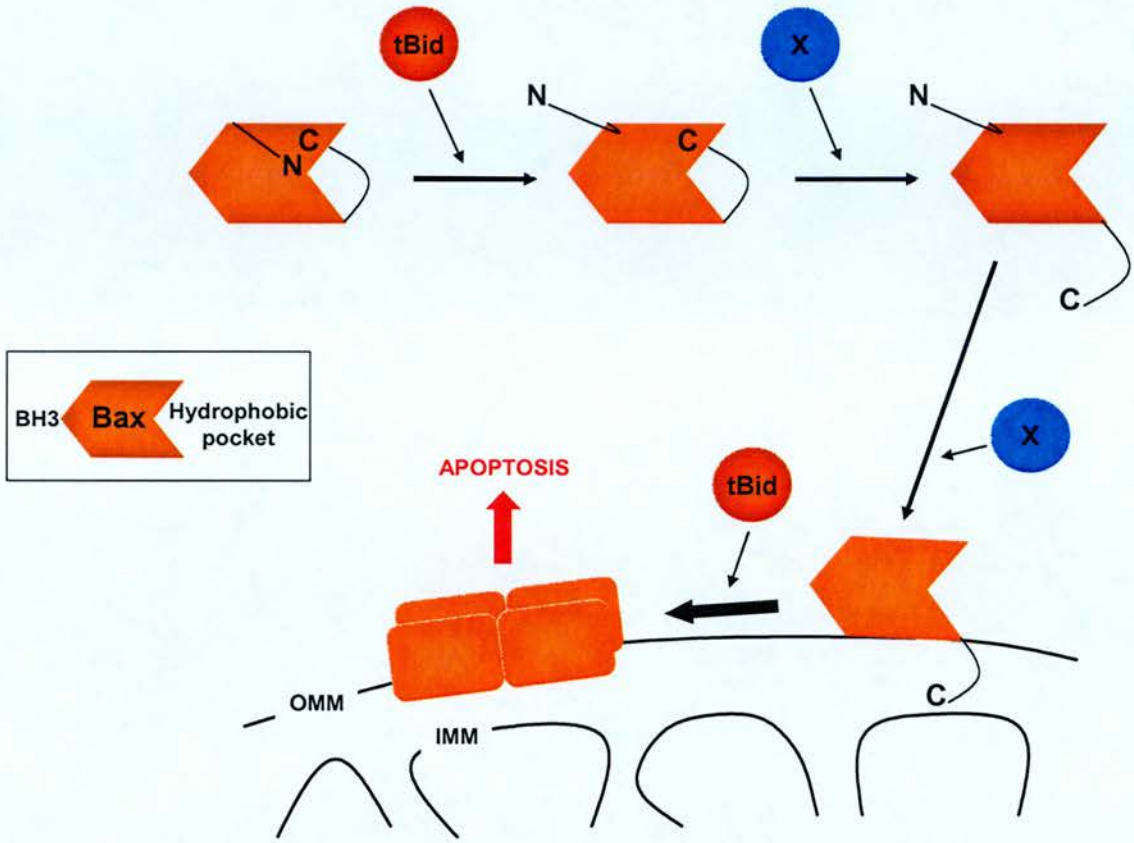

Figure. 1.12. A suggested model for the Bax activation sequence. Under resting conditions, Bax is predominantly cytosolic, with its C-terminal tail (TM domain) buried in a hydrophobic pocket formed by its BH1, BH2 and BH3 domains. Following stress-inducing signals, Bax undergoes conformational changes involving both the N- and C-terminal regions. This may be initiated through the binding of a BH3-only protein such as tBid or Puma, but may also involve as yet undetermined regulatory factors (represented by X). Release of the C-terminal TM domain from the BH3-binding pocket is thought to direct the translocation of Bax to mitochondria, a process that may also involve unknown chaperone factors (X). Accessibility of the BH3 binding pocket to other Bax BH3 domains has been suggested to lead to the homo-oligomerization of Bax molecules into predicted pore-like structures on the OMM, which has also been proposed to involve Bid/tBid. The formation of multimeric pores is one mechanism by which Bax may permeabilise the OMM and release cytochrome c to initiate downstream caspase activation. Image modified from Lucken-Ardjomande & Martinou, 2005.

1.11. Therapeutic potential of Bax-regulating proteins

Many diseases are associated with dysfunctional apoptosis (Thompson, 1995), and since the permeabilisation of the OMM is a central component in apoptotic pathways, the identification of new agonist/antagonists to Bax has enormous implications for the development of therapeutics targeting these diseases. The use of blood-brain barrier permeable caspase inhibitors to attenuate apoptosis associated with neurodegeneration has had limited success as mitochondrial dysfunction still occurs in the absence of caspases leading to impaired ATP production and the accumulation of reactive oxygen species (Chang *et al.*, 2002). Inhibiting caspase activity therefore appears to merely delay neuronal cell death rather than prevent it. As a result, efforts have instead focused on pathways upstream of mitochondrial dysfunction, in particular identifying pharmacologic agents capable of inhibiting the activation and translocation of Bax to the OMM (Polster and Fiskum, 2004). Two recently identified Bax-binding proteins, Humanin and Ku70, represent promising candidate therapeutic targets to limit Bax-mediated mitochondrial dysfunction. Humanin, a 34kDa peptide originally identified as a protective factor against neuronal cell death induced by amyloid-β protein, is a candidate template for the development of small molecule drugs to inhibit Bax-mediated apoptosis in neurodegenerative disorders (Guo *et al.*, 2003). The ablation of HN expression in glioblastoma cells by RNA interference has already been shown to increase their sensitivity to apoptosis induced by staurosporine and serum withdrawal (both mitochondria and thus Bax-dependent inducers of apoptosis) but not to mitochondria-independent insults, such as that induced by TRAIL (Guo *et al.*, 2003). Similarly, a protective effect against Bax-dependent stimuli was attributed to a cell-permeable five amino acid peptide derived from the Bax-binding domain of Ku70, known as Bax-inhibiting peptide (BIP), although the efficacy of this particular inhibitor has yet to be tested in neuronal cell lines (Sawada *et al.*, 2003). However, the identification of such Bax-regulatory factors also has important implications for developing new chemotherapeutic approaches. Following UV irradiation of 293T cells, Ku70 was shown to undergo acetylation at several C-terminal lysine residues which

induced the dissociation of the Bax-Ku70 complex (Cohen *et al.*, 2004). This has since led to the development of histone deacetylase inhibitors to induce Bax-mediated apoptosis in tumour cells, and are currently under clinical trial as potential adjuvants to cancer therapy treatments (Subramanian *et al.*, 2005). In light of this success, the search for additional Bax-binding proteins is intensifying.

1.12. Summary

Apoptosis is a highly regulated physiological process that is essential for the development and survival of all multicellular organisms. However, when the molecular mechanisms underlying this process falter, or when apoptosis is initiated within an inappropriate physiological setting, numerous disease states can arise, among them cancer, auto-immunity and neurodegeneration. The mammalian nervous system is particularly vulnerable to ischemic damage, in which apoptosis makes a significant contribution to the resultant neuronal cell loss. Apoptosis is also an active process contributing to the neuronal cell loss associated with neurodegenerative diseases. The activation and translocation of the pro-apoptotic Bcl-2 family protein, Bax, to mitochondrial membranes is a central component within stress-induced apoptotic pathways associated with disease pathology. The regulatory factors controlling Bax activation were, until recently, thought to be confined to other Bcl-2 family proteins. However, the elaborate conformational alterations that Bax needs to undergo during its activation process suggest that additional, non-related proteins, such as molecular chaperones, play a hand in regulating the bioactivity of Bax. Such regulatory factors therefore represent important new targets for therapeutic intervention.

1.13. Thesis hypothesis

The overall hypothesis of this thesis is that Bax resides in the cytosol of quiescent cells in a retentive protein complex, and that following receipt of an apoptotic stimulus, Bax dissociates from these retentive factors and engages with novel chaperone proteins that are required for Bax to translocate to the mitochondrial surface. This hypothesis will be tested in a neuronal cell model of the intrinsic apoptotic pathway using a proteomics-based approach.

1.14. Aims of the thesis

The aims of this thesis are four-fold:

1. To characterise the molecular events depicting the course of the intrinsic apoptotic pathway in an *in vitro* neuronal cell model of the intrinsic apoptotic pathway.
2. To identify Bax-binding proteins within a neuronal cell line in order to gain an insight into how Bax might be regulated under normal cellular conditions (the identification of Bax cytosolic retentive factors) and during apoptosis (identification of Bax translocation factors).
3. To characterise the interaction between Bax and identified binding proteins at the molecular level.
4. To evaluate the biological role of identified Bax-binding proteins using the established *in vitro* neuronal cell model of the intrinsic apoptotic pathway.

CHAPTER 2: General Materials & Methods

2.1. Chemicals & antibodies

All chemicals and antibodies were purchased from Sigma Aldrich Chemical Co. (Poole, UK) unless otherwise stated.

2.2. Cell culture

All culture dishes were from Gibco (Paisley, UK). SH-SY5Y human neuroblastoma cells were obtained from ECACC (No. 94030304) and grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-aldrich) supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were cultured in $165cm^3$ filter-capped flasks at 37°C in a 5% CO_2 atmosphere and split when approximately 80% confluent to limit overcrowding. Passage numbers between 12 and 18 were used for all experiments. For experimental analysis, cells were seeded at a density of 1×10^5 cell/well in 24-well culture dishes and allowed to attach for 24h prior to treatments. Apoptosis was induced using 500nM staurosporine (STS) (McGinnis *et al.*, 1999). Control cells received dimethyl sulphoxide (DMSO) vehicle (1:2000).

2.3. Extraction of whole cell lysates

For the collection of whole cell lysates, DMSO-vehicle or 500nM STS (5h)-treated SH-SY5Y cells were extracted in 150mM NaCl, 10mM HEPES-KOH (pH7.6), 1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS), 1mM phenylmethylsulphonylfluoride (PMSF), 10µg/mL each aprotinin, pepstatin and leupeptin; extraction buffer). Cells were homogenised using 10 'up/down' strokes in a tight-fitting glass Dounce homogeniser (Jencons, Leighton Buzzard, UK) and incubated on ice for 30mins. Lysates were then centrifuged at 13,000rpm in a micro-centrifuge to remove intact cells and cellular debri and supernatant retained. Protein concentration

was determined using a modified Lowry method (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories, Hemel Hempstead, UK) and lysates made up to 2mg/ml in extraction buffer. Lysates were stored at -70 C and thawed on ice when required.

2.4. Co-immunoprecipitation

Protein A-conjugated paramagnetic dynabeads (Invitrogen, Paisley, UK) were washed and equilibrated in extraction buffer and passed through the SH-SY5Y whole cell lysates to clear lysate of non-specific binding. Proteins were immunoprecipitated with a pan-reactive polyclonal Bax antibody (1:2000; Upstate Biotechnology, Lake Placid, NY, USA), a polyclonal Bax antibody raised against residues 43-61 within human Bax (1:500; Abcam, Cambridge, UK), a monoclonal Bax antibody recognising the conformational-specific 6A7 epitope (residues 12-24; BD Biosciences, Cowley, UK), a monoclonal nucleophosmin antibody raised against residues 1-68 within human nucleophosmin (5µg; Sigma) or a monoclonal survival motor neuron (SMN) antibody (5µg; BD Biosciences) overnight at 4˚C. Immune complexes were isolated by incubation with 20µl dynabeads for 2 hours at 4˚C with gentle agitation. Beads were collected by magnet and washed three times in extraction buffer before bound proteins were eluted by boiling in loading buffer (25mM Tris-HCL (pH 6.8), 0.8% SDS, 1% 2-mercaptoethanol, 4% glycerol, 0.01% bromophenol blue and 0.1M dithiothreitol (DTT)).

2.5. 1D SDS PAGE and Western blotting

Protein concentration was determined using a modified Lowry method (Bio-Rad Protein Assay Kit; Bio-Rad Laboratories) and 20µg of protein in loading buffer pipetted into each well of a 4-12% Tris-glycine gel (Invitrogen) and resolved by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for 40mins at 200mV. Proteins were then transferred to nitrocellulose membranes (Hybond C; Amersham Biosciences, Chalfont St Giles, Buckinghamshire, UK) for 1h at 30mV. Membranes

were incubated for 1h in blocking solution (50mM Tris-HCl, 150mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin. Membranes were then probed using the following antibodies: pan-reactive polyclonal Bax (1:2000), monoclonal nucleophosmin (1:1000), monoclonal β-actin (1:10,000), polyclonal Hsp60 (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal Hsp90 (1:1000; Santa Cruz Biotechnology), monoclonal Hsc71 (1:1000), monoclonal GRP78 (1:500), monoclonal β-tubulin (1:500), monoclonal vimentin (1:10,000), monoclonal VDAC (1:500; BD Biosciences), polyclonal survival motor neuron (SMN; 1:1000) and polyclonal growth factor receptor-bound protein 2 (GRB2; 1:5000; BD Biosciences) in blocking solution overnight at 4˚C. Membranes were washed 3 x 5 min in Tris Buffered Saline containing 0.05% Tween-20 (TBS-Tween) before incubation with horse radish peroxidase (HRP)-conjugated IgG in blocking solution for 1h at room temperature. Membranes were washed 3 x 5min in TBS-Tween and then detected with enhanced chemiluminescence (ECL plus; GE Healthcare, Chalfont St.Giles, UK). Chemiflourescence levels were measured using a FluorChem image analyser (AlphaInnotech, Braintree, UK). For quantification of protein expression, Nitrocellulose membranes were re-probed with a monoclonal antibody against the housekeeping protein β-actin, as described by Liao and colleagues (Liao *et al.*, 2000). Chemifluorescence was corrected for background, normalised to β-actin loading controls and expressed as a percentage change in expression compared to controls.

2.6. Confocal microscopy

For analysis by confocal microscopy, cells were cultured in glass bottomed 24-well culture plates (Barloworld Scientific, Aberbargoed, UK) or 35/22 mm diameter glass-bottomed poly L-lysine coated petri dishes (Willco Wells, Roysden, Herts, UK). Cells were washed in Hank's balanced salt solution (HBSS) and fixed in 4% paraformaldehyde-PBS for 10mins (for BaxNT antibody) or 10% formalin/methanol for 30mins (for nucleophosmin Sigma antibody) at room temperature. Cells were then gently rinsed three times in HBSS and permeabilised in ice-cold 100% methanol for

1min (for BaxNT antibody) or 2% CHAPS-PBS for 5mins at room temperature (for nucleophosmin Sigma antibody). Cells were blocked for 1h in 8% BSA before being incubated with a pan-reactive Bax antibody (BaxNT; 1:200) or a monoclonal nucleophosmin antibody (Sigma: 1:200) overnight at 4°C in 8% BSA solution. Cells were washed three times in HBSS containing 0.1% Tween-20 (HBSS-T) and incubated with 1μg/ml AlexaFluor fluorescent IgG antibodies (AlexaFluor-488 and ALexaFluor-546; Invitrogen) for 2h at room temperature in 8% BSA. Cells were rinsed three times in HBSS containing 0.05% Tween-20 before staining with the DNA intercalating dye TO-PRO-3 (1:1000; Invitrogen) for 5mins in HBSS followed by a final rinse in HBSS. Analysis was performed using a Zeiss META501 laser-scanning confocal microscope (Carl Zeiss Imaging, Welwyn Garden City, UK).

2.7. Statistical analysis

Unless otherwise stated, all data represents the mean of three independent experiments performed on separate days. Error bars, where appropriate, represent standard error of the mean (s.e.m). Statistical analysis was performed using SigmaStat v3.0 software (SPSS Inc., Woking, UK). Data were analysed by one-way or two-way analysis of variance (ANOVA) with *post-hoc* Dunnett's test where appropriate. Where data did not represent parametric distribution, Kruskal-Wallis one-way ANOVA was used with *post-hoc* Dunnett's test, where indicated. The significance level in all tests corresponds to a probability (p) value of less than 0.05 compared to a control group.

CHAPTER 3: Characterising the intrinsic apoptotic pathway

3.1. Chapter aims

In this chapter, an experimental neuronal cell model of the intrinsic apoptotic pathway will be established and characterised.

3.2. Introduction

The ischaemic environment of neural tissue affected by stroke and the toxic accumulation of protein aggregates in neuronal cells associated with neurodegenerative diseases stimulates activation of the intrinsic apoptotic pathway (Thompson, 1995; Mattson, 2000). In order to accurately study the biochemical events leading to apoptotic cell death in such disorders *ex-vivo*, two fundamental experimental requirements need to be met: 1) a relevant cell line that is genetically, physiologically and phenotypically comparable to human neuronal cells, and 2) an apoptotic stimulus that leads to the induction of the apoptotic pathway encountered within the *in vivo* setting.

The *in vitro* model chosen in this thesis was staurosporine (STS)-induced apoptosis in SH-SY5Y cells. The human neuroblastoma cell line, SH-SY5Y, has been widely used as a model in which to study neuronal cell death pathways (Boix *et al.*, 1997; McGinnis *et al.*, 1999; Kitamura *et al.*, 2003) and as an *in vitro* cellular model for Alzheimer's disease (Li *et al.*, 1996; Peraus *et al.*, 1997) and Parkinson's disease (Sheehan *et al.*, 1997; Fall and Bennett, Jr., 1999). The broad spectrum protein kinase inhibitor, staurosporine (STS) has been observed to induce apoptosis in SH-SY5Y cells that is caspase 8-independent (Lopez and Ferrer, 2000), involves Bax translocation (Desagher *et al.*, 1999; McGinnis *et al.*, 1999; Eskes *et al.*, 2000), mitochondrial cytochrome c release (Krohn *et al.*, 1999; Poppe *et al.*, 2001; Ahlemeyer *et al.*, 2002) and that can be inhibited by Bcl-X_L over-expression (Yuste *et al.*, 2002). Together, these observations

suggest that STS preferentially stimulates the intrinsic pathway to apoptotic cell death in this cell line.

3.2.1. The SH-SY5Y cell line as an *in vitro* model of neuronal cell apoptosis

The SH-SY5Y human neuroblastoma cell line is a thrice-cloned (SK-N-SH → SH-SY → SH-SY5 → SH-SY5Y) sub-line of the parental neuroepithelioma cell line, SK-N-SH, that was originally established in 1970 from the bone marrow biopsy of a four year old girl with metastatic neuroblastoma (Biedler *et al.*, 1973). Neuroblastoma is an extracranial solid tumour arising from malignant neuronal cells within the sympathetic nervous system and is the most common cancer affecting infants (Miller *et al.*, 1995). The resulting neuroblasts (comprising of malignant pluripotent sympathetic cells) invaginate and migrate along the neuroaxis during embryonic development and consequently can develop tumours in various sites, including the adrenal medulla, paraspinal ganglia, thorax and more distant sites such as pelvic and cervical regions (Jennings *et al.*, 1993). The derived SK-N-SH cell line, extracted from a bone marrow neuroblastoma, was of sympathetic adrenergic ganglion origin, and histological analysis confirmed the presence of three distinct cell types: 1) loosely attached, small, rounded neuronal N-type cells possessing many neuritic processes, 2) large, flattened and adherent epithelial/fibroblastic S-type cells, and 3) intermediate I-type cells (Ciccarone *et al.*, 1989).

In contrast to the parental SK-N-SH cell line, the derived SH-SY5Y cell line consists of a comparatively homogenous population of dopaminergic neuronal N-type cells which are morphologically and biochemically similar to sympathetic neurones (Ross *et al.*, 1983). When grown in culture, SH-SY5Y cells exhibit a neuroblast-like phenotype with short neurite-like processes and angular cell bodies containing large nuclei – see Figure 3.1. However, unless the cells are induced to terminally differentiate into a mature neuronal phenotype (which can be achieved with addition of retinoic acid, NGF or low doses of STS (Jensen, 1987; Jalava *et al.*, 1992)), serial passage of the cell line will

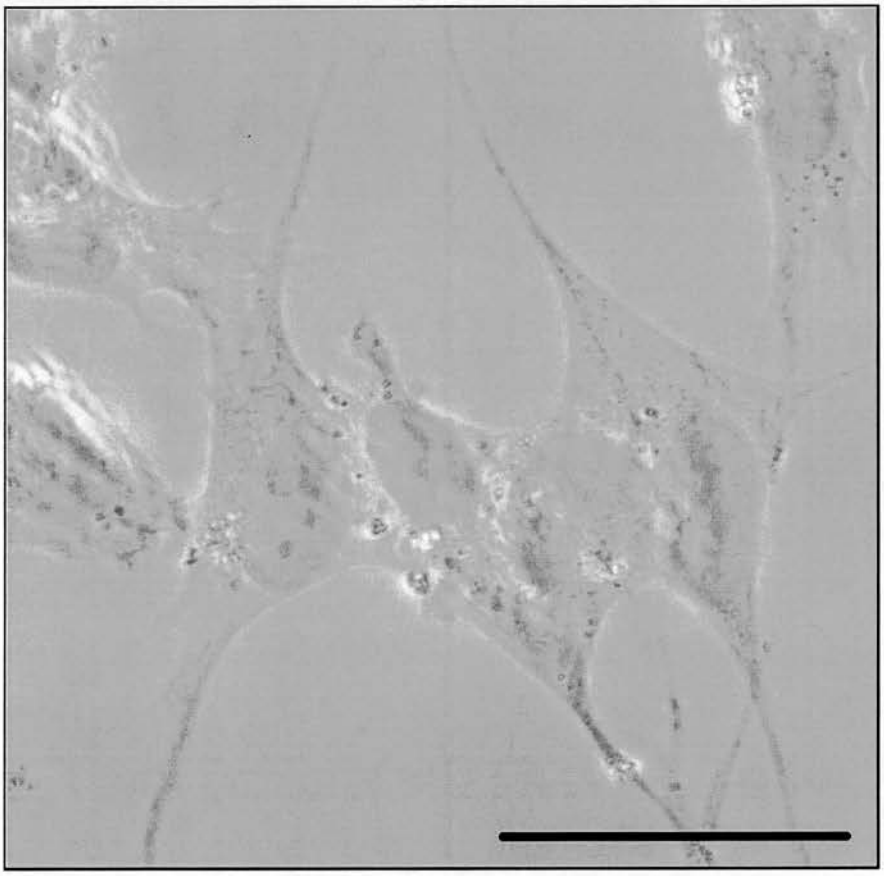

Figure 3.1. Phase-contrast image of undifferentiated SH-SY5Y cells. Cells typically have a large single nucleus containing one or two visible nucleoli. Undifferentiated cells have an average of 2-3 neuritic processes of 40-50 microns in length. Scale bar represents 50 microns.

eventually lead to the emergence of a predominantly epithelial phenotype (Ross *et al.*, 1983). Therefore, SH-SY5Y cells can only be reliably used as a neuronal cell model up to a passage number of 20 (European Collection of Cell Cultures (ECACC) recommendations).

3.2.2. Staurosporine as an inducer of the intrinsic apoptotic pathway

Staurosporine (STS) is a bacterial alkaloid produced by *Streptomyces staurospores* and is a potent and broad-spectrum inhibitor of protein kinases, such as protein kinase c (PKC) (Jarvis *et al.*, 1994), the cyclin-dependent kinases, CDK1 and CDK2 (Meijer, 1996), calcium/calmodulin-dependent kinase-2 (Yanagihara *et al.*, 1991) and receptor tyrosine kinases (Boix *et al.*, 1997). This shutdown in the cell's phosphorylation and signal-transducing machinery results in the induction of the intrinsic apoptotic pathway, as demonstrated by observing the translocation of Bax and the subsequent release of cytochrome c from mitochondria in a variety of cell lines exposed to STS (Desagher *et al.*, 1999; McGinnis *et al.*, 1999; Krohn *et al.*, 1999; Eskes *et al.*, 2000; Poppe *et al.*, 2001). Together, these data support the use of STS as an appropriate apoptotic inducer for investigating molecular events in experimental cell-based models of the intrinsic apoptotic pathway.

The concentration of STS applied to cells has important implications for its effect, particularly in the case of neuronal cells where STS has been observed to perform a dual function according to the concentration delivered. At low concentrations (100nM and below), STS induces neuritogenesis in a wide range of cultured neuronal cell lines, such as PC12 cells (Hashimoto and Hagino, 1989), dorsal root ganglia cultured from chick embryos (Sano *et al.*, 1994) and SH-SY5Y cells (Jalava *et al.*, 1992; Boix *et al.*, 1997). In SH-SY5Y cells, administration of 25nM STS promoted morphological and biochemical changes associated with the differentiation into mature neurones, such as extensive neurite outgrowth, and increased expression of the mature peripheral neurone markers, neuropeptide Y (NPY) and tyrosine hydroxylase (TH), as well as the growth-

associated protein 43 (GAP-43) which is specifically associated with the formation of axonal processes (Jalava *et al.*, 1992). Although STS was shown to inhibit PKC in these cells, other studies have shown that low dosages can activate certain growth-promoting kinases (Pavlovic-Surjancev *et al.*, 1993; Rasouly and Lazarovici, 1994) and stabilise the axonal microtubule assembly protein, Tau (Rasouly *et al.*, 1994), which may account for the neurotrophin-like effects observed at these concentrations (Boix *et al.*, 1997).

However, STS concentrations ranging from 50nM up to 1μM can induce apoptosis in a wide range of cells, including Jurkat T cells (Takayama *et al.*, 1995), HL-60 cells (Bertrand *et al.*, 1994), HeLa cells (Tafani *et al.*, 2001), cultured hippocampal neurons (Krohn *et al.*, 1999; Poppe *et al.*, 2001) and the neuroblastoma cell lines, NB69 and SH-SY5Y (Boix *et al.*, 1997; McGinnis *et al.*, 1999), indicating that there is some overlap in the concentrations at which STS induces differentiation or cell death, and that cell-type may also be a contributing factor to the outcome. One striking example of the latter emerged from a study of STS-induced cell death in a variety of neuroblastoma cell lines where exposure to increasing concentrations of STS triggered apoptotic cell death in NB69 and SH-SY5Y lines but not in IMR-5 or IMR-32 lines (Boix *et al.*, 1997) – see Figure 3.2.

A dose-response study of STS in SH-SY5Y cells demonstrated an EC_{50} for apoptosis induction of approximately 100nM (Boix *et al.*, 1997). Although addition of the pan-caspase inhibitor, z-VAD.fmk, was able to protect SH-SY5Y cells against STS-induced apoptosis at STS concentrations up to 500nM, at higher concentrations cell death escalated as a consequence of extensive oxidative damage (Yuste *et al.*, 2002). Taken together, these observations suggest that for induction of apoptosis in SH-SY5Y cells, STS concentrations between 200nM and 500nM are most efficacious.

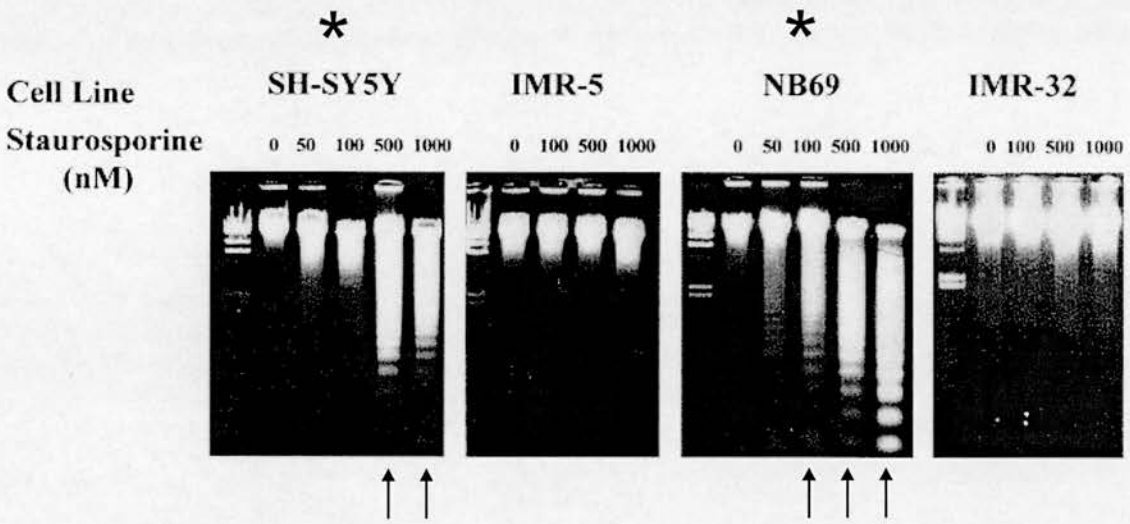

Figure 3.2. STS-induced apoptosis in a variety of human neuroblastoma cell lines. Only in SH-SY5Y and NB69 cell lines (*) was the characteristic pattern of inter-nucleosomal DNA fagmentation (DNA laddering; marked by arrows) observed using agarose gel electrophosrises following exposure to varying concentrations of STS. Image modifed from Boix et al, 1997.

3.3. Materials & Methods

3.3.1. Cell culture

SH-SY5Y human neuroblastoma cells were maintained as described in general materials & methods (Chapter 2). For experimental analysis, cells were seeded at a density of at 1 x 10^5 cells per well in 24-well tissue culture plates (Gibco) and allowed to attach for 24h prior to treatment. Apoptosis was induced by adding 500nM STS to culture medium. Control cells received DMSO vehicle (1:2000).

3.3.2. Assessment of Bax conformational change and translocation

Bax conformational change was assessed by immunoprecipitating endogenous Bax from control and apoptotic whole cell lysates (500nM STS, 5h) in extraction buffer using an antibody recognising an epitope within the N-terminus of Bax (6A7), as described in general materials & methods (Chapter 2). Briefly, vehicle and STS-treated SH-SY5Y whole cell lysates were immunoprecipitated with 5μg mouse monoclonal Bax 6A7 antibody and protein/antibody complexes anchored to protein-A coated dynabeads. Bound protein was eluted by boiling in loading buffer and the presence of 6A7-reactive Bax detected by Western blotting using a pan-reactive Bax antibody, as described in general materials & methods (Chapter 2).

The translocation of Bax to mitochondria during apoptosis was assessed by subcellular fractionation/Western blotting and by confocal microscopy. For analysis of Bax translocation by subcellular fractionation, cells were collected and contaminating media removed by two cycles of centrifugation (200g/7min) and re-suspension in HBSS. The resultant cell pellet was re-suspended in 10mM Tris-Cl (pH 6.7), 0.25M sucrose, 1mM EGTA, 10mM KCl, 0.15mM $MgCl_2$, 0.1mM DTT, 1mM PMSF, 20μM lactacystin, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM sodium orthovanadate and 10μg/mL each aprotinin, pepstatin and leupeptin (SCF buffer) and homogenised

using 10 'up/down' strokes in a tight-fitting glass Dounce homogeniser (Jencons). Nuclei, insoluble material and unbroken cells were removed by centrifugation for 3 min at 600g. An aliquot of the supernatant was removed and CHAPS added to a final concentration of 2%. This fraction was designated the whole cell lysate. The remaining supernatant was separated into crude cytosolic (supernatant) and mitochondrial (pellet) fractions by centrifugation (6,000g for 15 min). The crude mitochondrial fraction was then washed twice (6,000g for 15 min) with SCF buffer and the resultant pellet re-suspended in SCF buffer containing 2% CHAPS. This fraction represented an enriched-mitochondrial fraction. The crude cytosolic fraction was centrifuged at 100,000g for 1h with the resultant supernatant representing the soluble S100 cytosolic fraction. All fractionation procedures were performed at 4°C. Fractions were separated by 1D SDS-PAGE and analysed by Western blot for Bax, β-actin and VDAC expression, as described in general materials & methods (Chapter 2).

For analysis of Bax translocation by confocal microscopy, SH-SY5Y cells were seeded in glass-bottomed 24-well culture plates and allowed to attach for 24h before exposure to 500nM STS for 1h, 3h or 5h. For analysis of Bax co-localisation with mitochondria, vehicle-treated and STS (5h;500nM)-treated cells were incubated with culture medium containing 100nM MitoTracker Orange CMTMRos (Invitrogen) for 15mins under normal growth conditions prior to fixation in 4% paraformaldehyde-PBS. Cells were then fixed, stained and analysed by confocal microscopy as described in general materials & methods (Chapter 2).

3.3.3. Assessment of cellular morphology

The appearance of apoptotic cell morphology over a 5h timecourse of exposure to STS was assessed qualitatively by light microscopy. Live cells in culture were viewed under Hoffman illumination with a 40X objective (Nikon Instruments, Kigston, Surrey, UK) and photographed with a digital camera. Progression of apoptotic cell death was evaluated by monitoring the occurrence of morphological apoptotic hallmarks, such as

rounding of cell bodies, shrinkage of cell volume, condensation of nuclear structures and the eventual disintegration of nuclei into several membrane-bound fragments (Bellamy *et al.*, 1995).

3.3.4. Measurement of cytosolic cytochrome c

Cytosolic cytochrome c was measured using an in-house validated enzyme-linked immunosorbent assay (ELISA). . SH-SY5Y cells were cultured in 24-well culture plates and exposed to STS over a 5h timecourse. Cell lysis was then performed using 150mM NaCl, 10mM HEPES-KOH (pH7.6), 1mM PMSF, 10μg/mL each aprotinin, pepstatin and leupeptin (lysis buffer) containing either 3.125μM digitonin to extract cytosolic fraction or 3.125μM digitonin + 1% CHAPS to extract whole cell lysate for one hour at room temperature. Lysates were centrifuged at 10,000g for ten minutes to remove nuclei and unbroken cells. Supernatants were serially diluted (1:3, 1:9, 1:27) in lysis buffer and cytosolic cytochrome c content measured using a commercially available ELISA (Caltag MedSystems, Botolph Claydon, UK) according to the manufacturer's instructions. Briefly, 96 well plates were coated with capture antibody (purified mouse anti-human cytochrome c monoclonal antibody; 1μg/ml), sealed and incubated overnight at 4°C. Plates were then washed three times with wash buffer and blocked with assay diluent for 2h at room temperature. After a further three wash cycles with wash buffer, 100μl standards, samples and blanks were added to separate wells, then 50μl biotin conjugated mouse anti-human cytochrome c antibody and plates incubated for 2h at room temperature. Plates were washed three times with wash buffer and bound complexes detected using goat anti-rabbit HRP antibody (1h at room temperature) and TMB substrate solution (30min in the dark). The reaction was stopped by addition of 1M Phosphoric acid and absorbance read at 450nm within 30min. Cytosolic cytochrome c (measured in digitonin-containing extraction buffer) was calculated as a percentage of the total cellular content (measured in digitonin + CHAPS containing extraction buffer) for each time point analysed.

3.3.5. Measurement of cleaved caspase-3

SH-SY5Y cells were cultured in 24-well culture plates and exposed to STS over a 5h timecourse. Cell lysates were extracted in lysis buffer containing 1% CHAPS as described above. Active human caspase-3 was detected by ELISA (BD Biosciences) according to the manufacturer's instructions. Briefly, 96-well plates were coated with capture antibody (mouse anti-human caspase-3 monoclonal antibody; 1:240), sealed and incubated overnight at 4°C. Plates were then washed three times with PBS containing 0.1% Tween 20 (PBS-T) and blocked with assay diluent for 1h at room temperature. After a further three wash cycles with PBS-T, standards, samples and blanks were added to plates and incubated for 2h at room temperature. Plates were then washed five times in PBS-T before detection antibody (rabbit anti-cleaved caspase-3 monoclonal antibody; 1:240) was added and incubated a further 1h. After washing five times in PBS-T, bound complexes were detected using goat anti-rabbit HRP antibody (1h at room temperature) and TMB substrate solution (30min in the dark). The reaction was stopped by addition of 2N sulphuric acid and absorbance read at 450nm within 30min. Caspase-3 activity was calculated as a percentage of active caspase-3 in apoptotic (4μM camptothecin; 4h) Jurkat cell lysate standards provided with the ELISA kit.

3.3.6. Detection of DNA fragmentation

SH-SY5Y cells were cultured in 24-well culture plates and exposed to STS over a 5h timecourse Occurrence of internucleosomal DNA fragmentation was assessed quantitatively by performing terminal deoxynuceotidyl transferase-mediated dUTP nick end labelling (TUNEL) analysis with a commercially available ELISA (Roche Diagnostics Ltd, Burgess Hill, UK) according to the manufacturer's instructions. Briefly, cells were washed in HBSS and incubated with 200μl Roche kit lysis buffer for 30mins at 37°C, 5% CO_2. Lysates were then transferred into micro centrifuge tubes and centrifuged at 200g to remove cellular debris and intact cells. Supernatants were diluted 1:10 before being analysed for the presence of cytoplasmic histone-associated DNA

fragments (mono- and oligo-nucelosomes) by ELISA according to the manufacturer's instructions. Briefly, 20µl lysates, controls and blanks were added to wells of a streptavidin-coated 96-well plate. 80µl immunoreagent (biotin-conjugated mouse anti-human histone monoclonal antibody + peroxidase-conjugated mouse anti-human DNA monoclonal antibody) was then added to each well and plates sealed and incubated on a shaker (300rpm) for 2h at room temperature. Plates were washed three times with incubation buffer before detection of captured complexes with ABTS solution. Absorbance was read after sufficient colour change (10-20min) at 405nm.

3.3.7. Measurement of cell viability

SH-SY5Y cells were cultured in 24-well culture plates and exposed to STS over a 5h timecourse. Cell viability was measured using a commercial MTS assay (Cell Titer 96® Aqueous One Solution Assay; Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, cells were rinsed three times with warm HBSS and replenished with fresh culture media containing a 1:5 dilution of MTS reagent. Cells were incubated at 37°C in a 5% CO_2 atmosphere until sufficient colour had developed (typically 1-4hrs). 20µl aliquots (in triplicate) were removed from each well and transferred to a 96-well plate and absorbance read at 490nm.

3.4. Results

3.4.1. STS activates the intrinsic apoptotic pathway in SH-SY5Y cells

SH-SY5Y cells were exposed to 500nM STS for 5h and the induction of a Bax-directed apoptotic pathway assessed by measuring the activation and translocation of Bax to the mitochondrial surface. Furthermore, a 5h time course of STS treatment was performed and the progression of apoptosis measured by analysing Bax translocation, apoptotic cell morphology, the release of cytochrome c from mitochondria, caspase-3 activity, DNA fragmentation and cell death.

The presence of activated Bax in STS-treated SH-SY5Y cells was assessed by immunoprecipitating Bax from control and apoptotic SH-SY5Y lysates with a conformation-sensitive antibody recognising the 6A7 epitope – see Figure 3.3a. A small amount of 6A7-reactive Bax in vehicle-treated lysates was detected. Following 5h exposure to STS, however, the amount of activated Bax in the cell lysate was markedly increased.

The shift in distribution of Bax from the cytosol to the surface of mitochondria in response to STS treatment was assessed by subcellular fractionation and Western blotting using a pan-reactive, conformation-independent antibody – see Figure 3.3b. Under control conditions, Bax was primarily cytosolic but became markedly more abundant in the mitochondrial fraction of cells exposed to 5h STS. This translocation event was visualised using confocal microscopy – see Figure 3.3c. Endogenous Bax under normal (control) conditions appeared predominantly cytosolic as indicated by diffuse intracellular staining. Following 5h exposure to STS, however, Bax had undergone translocation to the mitochondrial surface as evidence by its punctuate mitochondria-like staining pattern. A time-course of Bax translocation was also performed which demonstrated the appearance of a punctate staining pattern by 1h exposure to STS – see Figure 3.4. Double-labelling with a mitochondria-specific stain

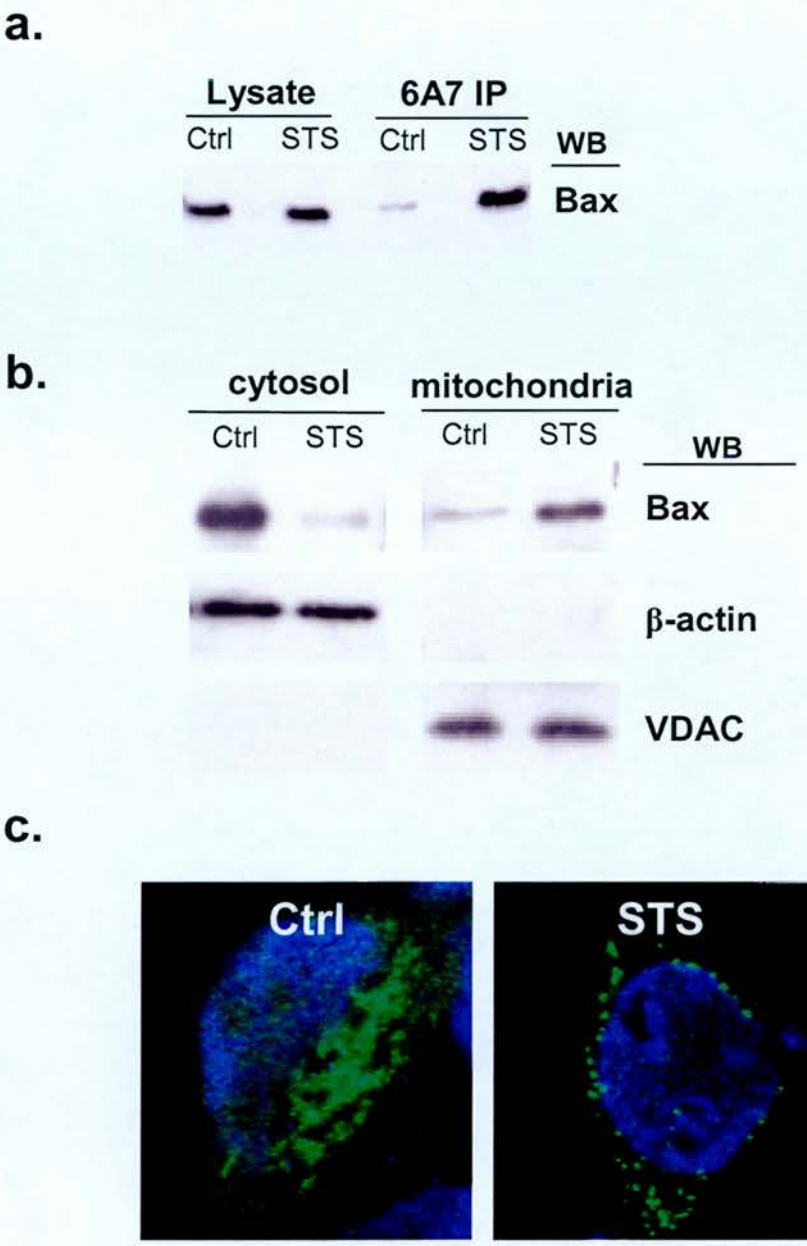

Figure 3.3. STS induces Bax activation and translocation. Exposure of an N-terminal 6A7 epitope in response to STS treatment allows Bax to be immunoprecipitated by a 6A7-reactive antibody **(a)**. Subcellular fractionisation and Western blotting using a pan-reactive, conformational-independent antibody **(b)** demonstrates re-distribution of Bax from the cytosol to mitochondria. β-actin and voltage-dependant anion channel (VDAC) represent cytosolic and mitochondria-specific proteins, respectively. Confocal microscopy **(c)** illustrates diffuse cytosolic Bax (green) under normal conditions (Ctrl) and mitochondria-associated punctate staining in apoptotic cells following 5h exposure to STS. Nuclei were pseudostained blue using TO-PRO-3. Ctrl, control cells; STS, cells exposed to 5h 500nM STS; WB = western blot. Data represent 3 independent experiments.

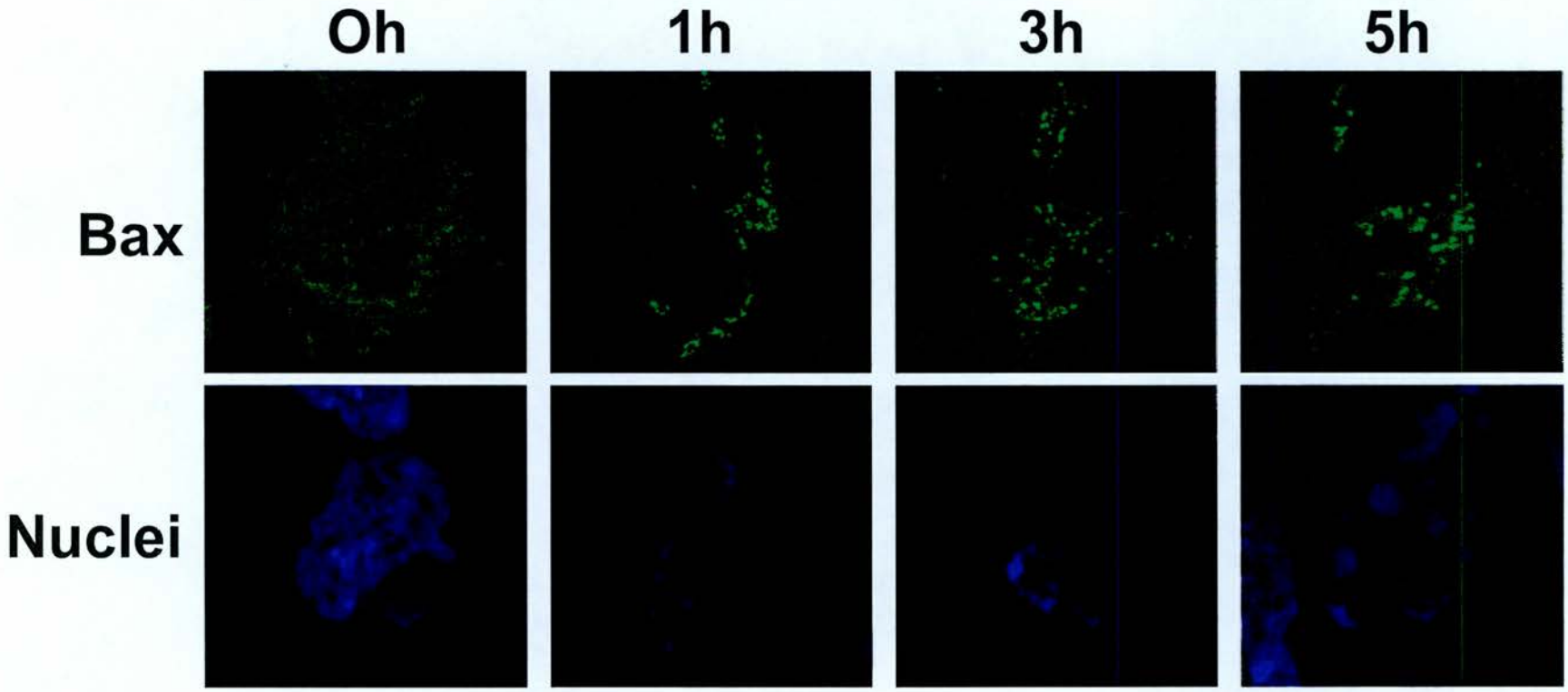

Figure 3.4. Bax translocation timecourse in STS-treated SH-SY5Y cells. Bax translocation was assessed by confocal microscopy (1000X magnification) during a 5h STS treatment timecourse. In control cells (0h), Bax (FITC; green) was cytosolic as indicated by the diffuse staining pattern. After 1h exposure to STS, however, Bax staining had become punctate indicative of mitochondrial relocalisation. Nuclei in the bottom panels are pseudo-stained blue. Images shown are representative of three independent experiments.

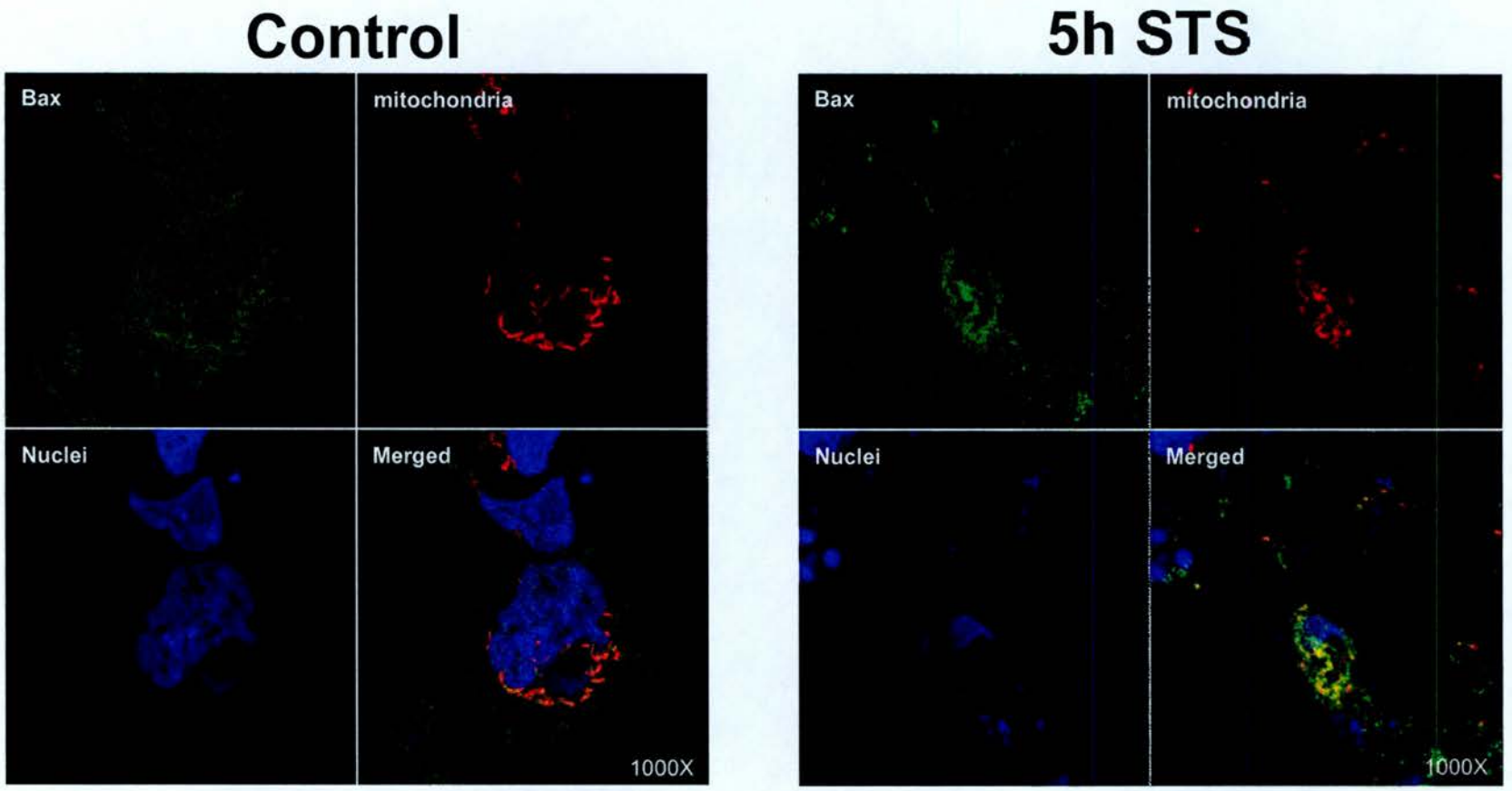

Figure 3.5. Bax co-localises with mitochondria in SH-SY5Y cells exposed to STS. In vehicle-treated cells **(Control)** Bax (green) displays a diffuse cytosolic staining pattern. Following 5h exposure to STS **(5h STS)**, however, Bax staining is punctate and co-localises with mitochondria (red). Yellow staining indicates co-localisation between Bax and mitochondria. Nuclei are pseudo-stained blue. Images shown are representative of three independent experiments.

confirmed that Bax had undergone relocalisation to mitochondria by 5h exposure to STS – see Figure 3.5.

Isolation and measurement of cytosolic cytochrome c content in SH-SY5Y cells was achieved by selectively permeabilising the plasma membrane with a lysis buffer containing 3.125μM digitonin. Previous work by Kirchoff & colleagues determined 3.125μM as the optimal concentration of digitonin detergent that would permeabilise the plasma membrane but leave mitochondrial and other organelle membranes intact (Kirchhoff *et al.*, 2002). A pilot study to determine if this approach was suitable for characterising cytochrome c release in STS-treated SH-SY5Y cells was performed by comparing cytosolic cytochrome c levels in control and apoptotic (STS; 5h) lysates extracted in buffers containing either 3.125μM digitonin or 3.125μM digitonin + 1% CHAPS. The addition of CHAPS detergent ruptured all cellular membranes and permitted the measurement of total cellular cytochrome c content, as illustrated in Figure 3.6a. With the CHAPS-containing extraction buffer, no differentiation could be made between cytochrome c content in untreated and apoptotic lysates as both readings equated to the total cellular content - see Figure 3.6b. With the extraction buffer containing digitonin alone, however, the change in distribution of cytochrome c from within mitochondria under normal conditions to its accumulation in the cytosol following exposure to STS was readily detected - Figure 3.6b. The 5h time-course profile of cytochrome c release from mitochondria in STS-treated SH-SY5Y cells was then measured by ELISA. An significant increase in cytosolic cytochrome c was detectable by 0.5h exposure to STS ($P = <0.05$) and increased to reach a maximal release of 70% (±9%) total cellular content by 4h – see Figure 3.7.

The five hour time course profile of procaspase-3 cleavage in STS-treated SH-SY5Y cells was also measured by ELISA. A significant increase in procaspase-3 cleavage was detected by 1.5h exposure to STS ($P = <0.05$) and increased two fold within 30mins, reaching a maximal level by 3h - see Figure 3.8.

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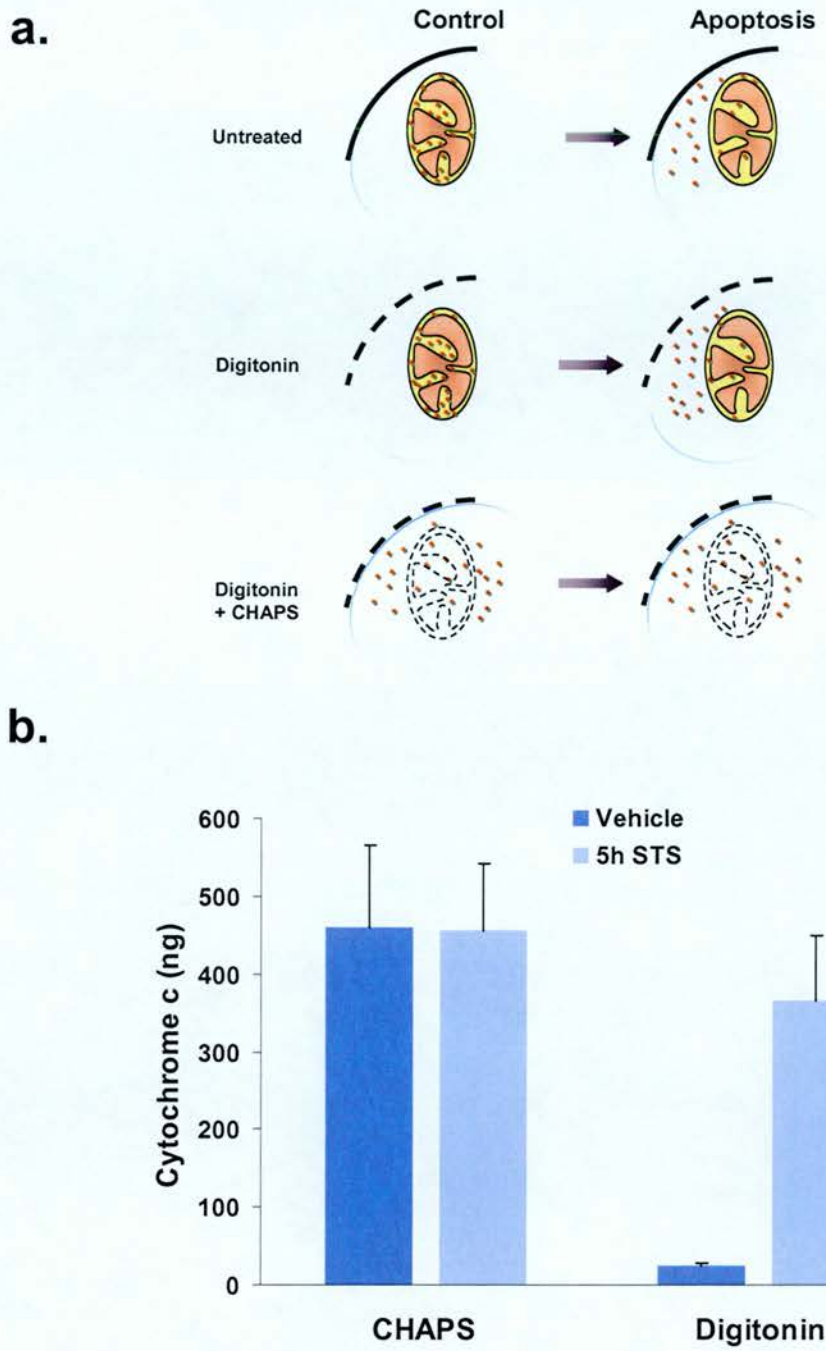


Figure 3.6. Isolation of cytosolic cytochrome c by selectively permeabilising the plasma membrane with 3.125μM digitonin. Selective lysis of the plasma membrane using digitonin allows comparison between cytosolic and mitochondrial cytochrome c pools, whereas addition of 1% CHAPS results in lysis of all cellular membranes **(a)**. Cytochrome c in cell lysates extracted from vehicle-treated or 5h STS-treated cells using buffers containing 3.125μM digitonin ± 1% CHAPS was measured by ELISA **(b)**. Addition of CHAPS resulted in quantification of total cellular cytochrome c whereas extraction with digitonin alone allowed measurement of cytosolic cytochrome c. Error bars represent s.e.m of three independent experiments.

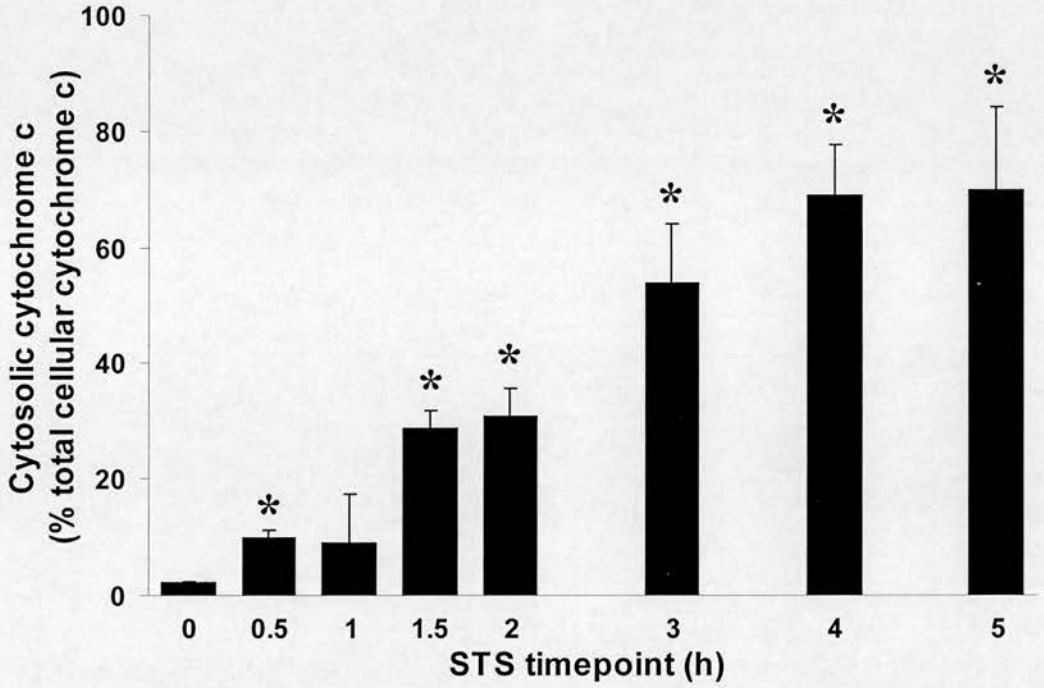


Figure 3.7. STS induces cytochrome c release. Cytosolic cytochrome c in SH-SY5Y cells exposed to a 0-5h timecourse of STS treatment was measured by ELISA according to the modified protocol described in materials & methods. Cytochrome c was detectable in the cytosol by 0.5h and reached a maximal plateau at 3-5h post-stimulus. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way ANOVA with post hoc Dunnett's test. * = $p<0.05$ versus 0h control.

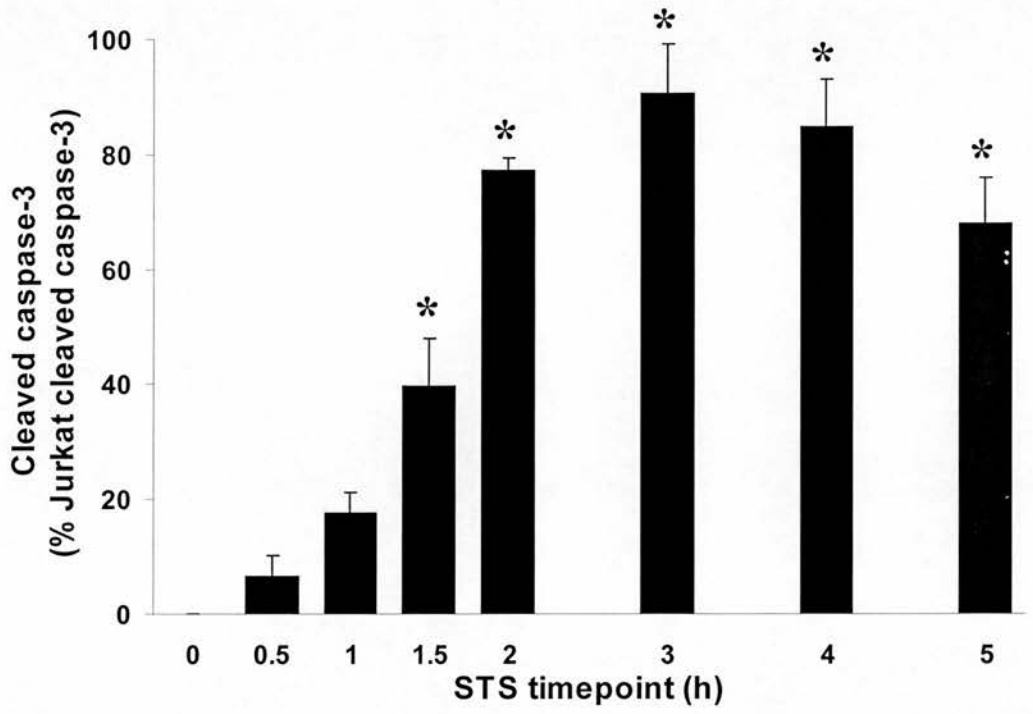


Figure 3.8. STS activates caspase-3 in SH-SY5Y cells. Casapse-3 in SH-SY5Y cell lysates exposed to a 0-5h time course of STS treatment was measured by ELISA. Casapse-3 activity was detectable by 1.5h and reached a maximal level by 3h exposure to STS. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way ANOVA with post hoc Dunnett's test. * = $p<0.05$ versus 0h control.

The emergence of apoptotic cell morphology in STS-treated SH-SY5Y cells was examined by light microscopy – see Figure 3.9. By one hour following exposure to STS, no observable changes to cell morphology had taken place. By three hours, however, evidence of cell rounding and shrinkage were apparent – see Figure 3.9a - and the appearance of morphological features associated with caspase activity, such as membrane blebbing and dense, fragmented nuclear structures (Wyllie *et al.*, 1980) were detectable by 4h exposure to STS – see Figure 3.9b.

The biochemical apoptotic hallmark of internucleosomal DNA fragmentation was assessed quantitatively during STS-induced apoptosis by a TUNEL-based ELISA. A significant increase in DNA fragmentation was detectable by 3h exposure to STS ($p = <0.05$) – see Figure 3.10.

A significant decrease in SH-SY5Y cell viability during STS treatment, as assessed by MTS assay, was apparent by 3h exposure to STS ($p = <0.05$), falling below 50% by 4h and reaching approximately 20-30% control levels by 5h post-treatment – see Figure 3.11.

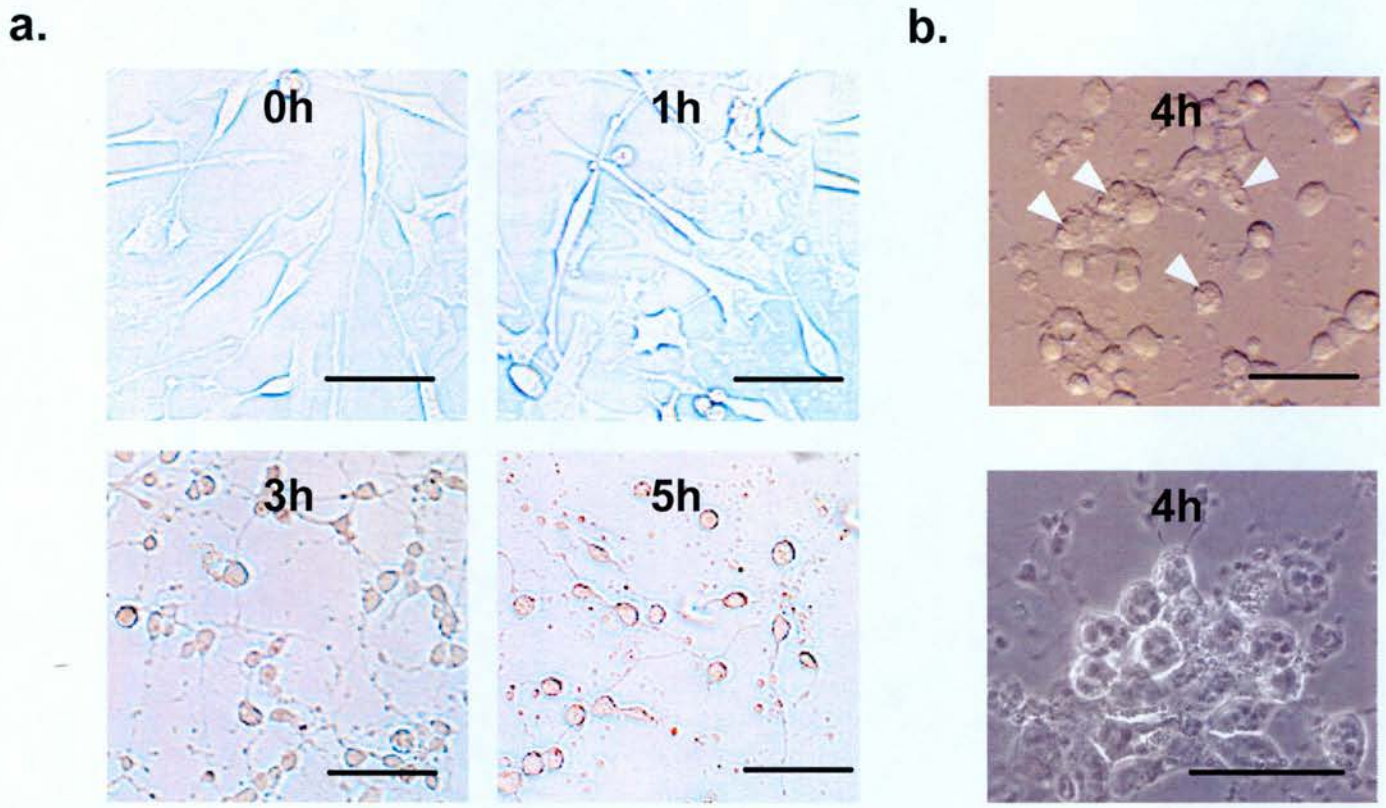


Fig. 3.9. STS-induced apoptotic cellular morphology. SH-SY5Y cells were exposed to 500nM STS for up to five hours and the appearance of apoptosis-related morphological changes recorded by light microscopy. **(a)** Morphological evidence of apoptotic cell death was apparent by 3h exposure to STS, with rounding of cell bodies, shrinkage of cell volume and detraction of neuritic processes evident. The emergence of specific apoptotic morphological features by 4h STS exposure is shown in **(b)**: plasma membrane blebbing (indicated by arrows; top panel) and fragmentation of nuclei (bottom panel). Scale bars represent 100 microns.

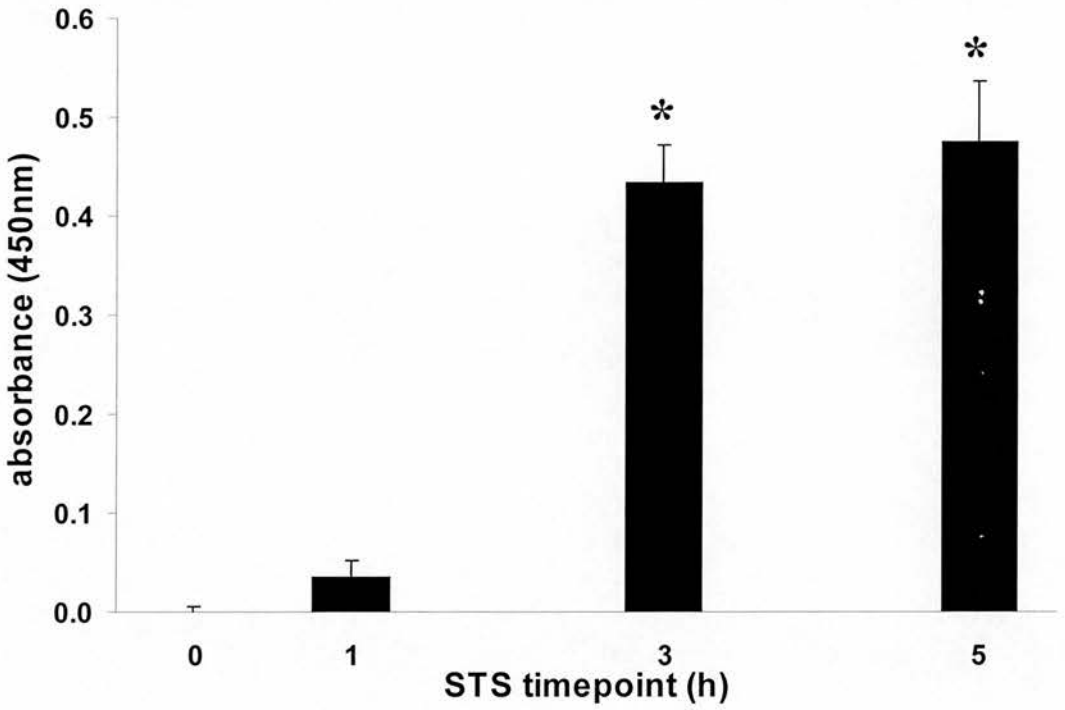


Figure 3.10. STS leads to internucleosomal DNA fragmentation as measured by TUNEL ELISA. DNA fragmentation in SH-SY5Y cells was detectable by 3h during exposure to STS. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way ANOVA with post hoc Dunnett's test. * = $p<0.05$ versus 0h control.

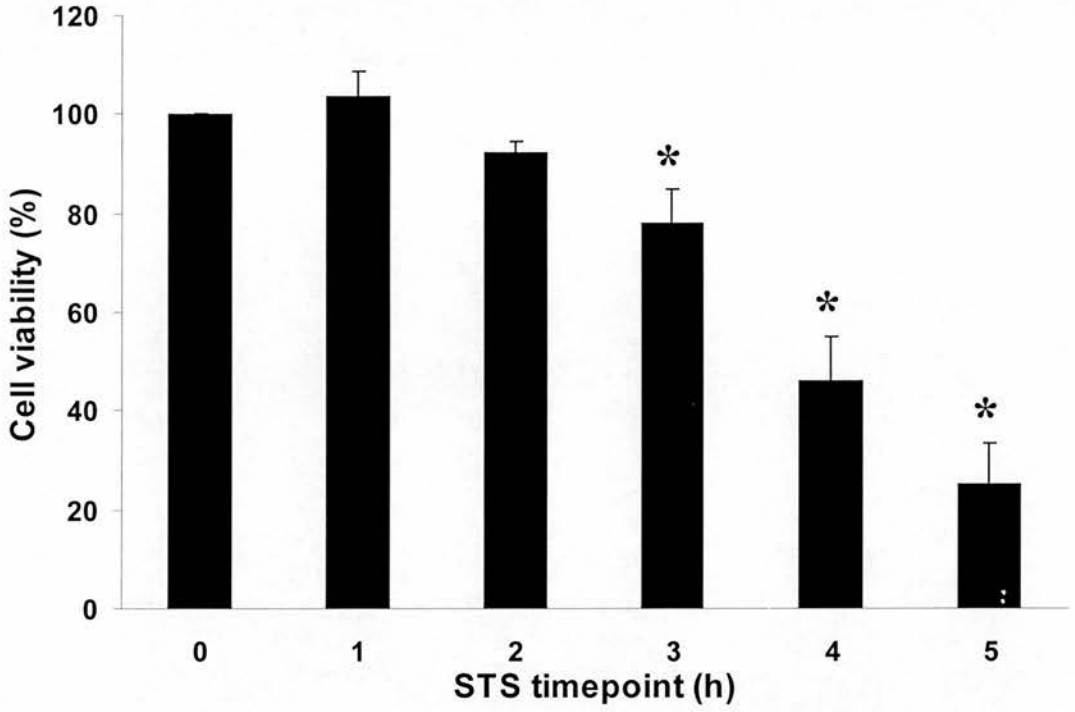


Figure 3.11. STS leads to cell death. A significant reduction in SH-SY5Y cell viability, as assessed by MTS assay, was detectable by 3h during exposure to STS and reached approximately 25% control levels by 5h. Data represent mean + s.e.m of four independent experiments. Data were analysed by one-way ANOVA with post hoc Dunnett's test. * = $p<0.05$ versus 0h control.

3.5. Discussion

Activation of the intrinsic apoptotic pathway is thought to be the principle pathway leading to apoptotic cell death in a variety of neurodegenerative disorders (Thompson, 1995; Mattson, 2000). Hence, when modelling the physiological apoptotic cell death associated with neurodegenerative disorders *in vitro*, it is important to confirm that an appropriate signalling pathway is active within the chosen model system. The intrinsic apoptotic pathway is characterised by a series of distinct biochemical events that can be considered as measurable molecular hallmarks. These include the activation and translocation of the pro-apoptotic Bcl-2 family protein, Bax, to the mitochondrial surface (Wolter *et al.*, 1997), the release of cytochrome c across the OMM into the cytosol (Liu *et al.*, 1996) and the downstream activation of caspases leading to apoptosis (Zou *et al.*, 1997; Li *et al.*, 1997). However, the signal transduction pathways leading to apoptosis differ according to the nature of the stress-inducing stimulus as well as the cell type (Bossy-Wetzel *et al.*, 1998; McGinnis *et al.*, 1999). Therefore, the intrinsic pathway-specific biochemical markers described above were used to confirm that STS treatment of SH-SY5Y cells induces the intrinsic apoptotic pathway.

Since the main focus of this thesis is to gain a better understanding of how the activation and translocation of Bax is regulated, it was important to confirm that STS initiated this process in SH-SY5Y cells. Bax activation is characterised by conformational alterations that expose previously hidden regions within both the N- and C-terminal domains (Goping *et al.*, 1998; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000). Immunoreactivity against one of these regions, the 6A7 epitope within the Bax N-terminus, is an established experimental marker for activated Bax in a variety of cell lines under different apoptosis-inducing conditions (Hsu and Youle, 1997; Nechushtan *et al.*, 1999; Sawada *et al.*, 2003). In this study, 6A7 immunoreactive Bax was immunoprecipitated in SH-SY5Y lysates exposed to STS, with only a residual amount detectable in lysates extracted from control cells. These data therefore suggest that STS induces Bax activation within the chosen cellular model. However, exposure of the 6A7 epitope has

been reported to occur independently of Bax translocation to mitochondria (Nechushtan *et al.*, 1999; Makin *et al.*, 2001). The translocation of Bax to the OMM has previously been observed in many cell types undergoing apoptosis (Wolter *et al.*, 1997; Hsu *et al.*, 1997b; Goping *et al.*, 1998) including neuronal cells (McGinnis *et al.*, 1999; Makin *et al.*, 2001) and is a central event within the intrinsic apoptotic pathway. Therefore a separate measurement of the translocation event was required to confirm that the apoptosis induced by STS utilised Bax. Using subcellular fractionation, Western blotting and confocal microscopy, endogenous Bax was observed to undergo re-distribution from a cytosolic to a mitochondria-associated localisation in response to STS treatment. Moreover, this event was observed to have taken place by 1h exposure to STS, in agreement with previous findings that Bax translocation is an early event within neuronal cell apoptosis (McGinnis *et al.*, 1999; Putcha *et al.*, 1999). Thus, STS induces the conformational changes and translocation event associated with the apoptogenic activity of Bax.

The release of cytochrome c from mitochondria into the cytosol is specifically associated with apoptosis and is not observed during necrotic cell death (Goldstein *et al.*, 2000b). Furthermore, this event is considered to be a gold standard for the detection of apoptosis operating through the intrinsic pathway (Kluck *et al.*, 1997; Green and Reed, 1998). Cytochrome c release has been directly linked with permeabilisation of the OMM by Bax (Jurgensmeier *et al.*, 1998; Eskes *et al.*, 1998; McGinnis *et al.*, 1999), and the rate by which cytochrome c is released into the cytosol has been shown to be directly proportional to the amount of Bax arriving at the mitochondrial surface at any given time (McGinnis *et al.*, 1999). For these reasons, mitochondrial cytochrome c release was chosen as an early and reliable indicator that a Bax-dependent apoptotic pathway was progressing within SH-SY5Y cells in response to STS-treatment. A significant increase in cytosolic cytochrome c was detected as early as 0.5h following onset of STS treatment and reached a maximal level by 4h exposure to STS. Previous studies support the view that cytochrome c is released from mitochondria in two distinct phases due to the presence of soluble and membrane-attached fractions of cytochrome c within the

inter-mitochondrial membrane space (Luetjens *et al.*, 2001; Ott *et al.*, 2002). Although no evidence for such a bi-phasic release was found within this study, the time-course profile of mitochondrial cytochrome c release presented here is in agreement with previous observations made using the same STS concentration and cell line (Posmantur *et al.*, 1997; McGinnis *et al.*, 1999).

Since the release of cytochrome c from mitochondria leads (via the initiator caspase-9) to the activation of the principle effector caspase, caspase-3 (Liu *et al.*, 1996; Bossy-Wetzel *et al.*, 1998; McGinnis *et al.*, 1999), analysis of procaspase-3 cleavage in STS-treated SH-SY5Y cells was also included to further characterise the apoptotic events occurring downstream of Bax translocation. A significant increase in procapsase-3 cleavage was detected by 1.5h exposure to STS, placing this event downstream of mitochondrial cytochrome c release, as has previously been reported in this model system (McGinnis *et al.*, 1999). However, no basal caspase 3 activity was detected as has been previously reported for SH-SY5Y cells (Posmantur *et al.*, 1997), perhaps due to limitations in the sensitivity of our chosen approach. Although not representing a significant increase, some procaspase-3 cleavage was detected at 0.5h and 1h exposure to STS. In addition to the cytosol, a proportion of procaspase-3 is also present within the mitochondrial inter-membrane space (Mancini *et al.*, 1998) where its cleavage and release into the cytosol has been observed to occur concomitantly alongside cytochrome c in response to STS treatment (Samali *et al.*, 1999). This apoptosome-independent route to caspase-3 activation could therefore account for the small amount of procaspase-3 cleavage detected in this study prior to the 1.5h timepoint.

The morphological hallmarks of apoptotic cell death were first documented and reported by Kerr and colleagues in 1972 (Kerr *et al.*, 1972). Morphological analysis of the structural cellular changes taking place during apoptosis has been widely used since then as a method to characterise its progression and to distinguish this death pathway from that leading to necrotic cell death (Walker *et al.*, 1988; Goldstein *et al.*, 2000a). The first readily observable morphological changes to take place following induction of

apoptosis are rounding of the cell body and shrinkage of cell volume (Goldstein *et al.*, 2000a). In this study, exposure to STS induced cell rounding and shrinkage of SH-SY5Y cells by 3h, as observed using light microscopy. The majority of morphological changes associated with apoptosis, such as cell rounding, shrinkage, nuclear fragmentation and blebbing of the plasma membrane, are directed by caspase activity, particularly as a result of caspase-mediated cleavage of essential structural components such as cytoskeletal proteins (Janicke *et al.*, 1998a) The emergence of distinctive apoptotic morphology at 3h is consistent with the observed elevation in cytosolic cytochrome c levels by 0.5h and caspase-3 activation by 1.5h exposure to STS. Loss of plasma membrane integrity, characterised by the formation of large blebs (zeiosis), is regarded as one of the markers of late apoptosis, occurring downstream of phosphatidylserine (PS) exposure on the extracellular surface of the plasma membrane (Goldstein *et al.*, 2000a). The appearance of cell surface blebbing in STS-treated SH-SY5Y cells was observed by four hours, by which time active caspase-3 levels in these cells had reached their peak.

The internucleosomal fragmentation of genomic DNA was the first recognised biochemical marker of apoptosis (Wyllie, 1980) and continues to be used as a method to confirmation the presence of apoptosis in a cell population. In this study, the appearance of mono- and oligo-nucleosomes within STS-treated SH-SY5Y cell lysates was quantified using a TUNEL-based assay and was observed to correlate with maximal caspase-3 activity. This finding is in agreement with the role played by caspase-3 in directing DNA fragmentation via its cleavage of the nuclear factor ICAD/DFF45 into the active DNase primarily responsible for cleaving genomic DNA during apoptosis (Liu *et al.*, 1997; Enari *et al.*, 1998).

Finally, the demise in SH-SY5Y cellular viability during STS treatment was assessed by MTS assay. This assay relies on a cell's oxidative metabolism to bioreduce a MTS tetrazolium compound to a coloured formazan product that can then be measured empirically. This assay is therefore an indicator of mitochondrial function rather than

cell viability *per se*, but can be used, as it is here, to measure a cell's "metabolic viability." The protracted decrease in metabolic viability observed during STS exposure, in contrast to the more rapid escalation of apoptotic biochemical markers such as cytochrome c release and capsase-3 activation, is in agreement with the reliance of apoptotic pathways progression on a continued supply of ATP (Leist *et al.*, 1997). However, as cytochrome c continues to be released from the mitochondrial inter-membrane space, the electron transport chain component of the oxidative phosphorylation machinery eventually breaks down and mitochondrial ATP production ceases to function efficiently (Mootha *et al.*, 2001). Consequently, after 5h exposure to STS, metabolic activity, as assessed by MTS bioreduction, had diminished to approximately 20% normal levels.

3.6. Summary

From the analysis of such key biochemical and molecular events as Bax activation/translocation, mitochondrial cytochrome c release, caspase-3 activation, DNA fragmentation and the assessment of cellular morphological changes, a well characterised model of the intrinsic apoptotic pathway in STS-treated SH-SY5Y cells has been established. This cellular model will be used in the following chapters to 1) identify Bax-binding proteins (Chapter 4) and 2) determine the functional contribution of the novel Bax chaperone to the bioactivity of Bax (Chapter 6).

CHAPTER 4: Identification of Bax-binding proteins using a proteomics-based approach

4.1. Chapter aims

In this chapter, a proteomics-based approach will be employed to identify 1) proteins binding the endogenous Bax whole molecule and 2) proteins binding specifically to the Bax C-terminal transmembrane domain within the SH-SY5Y cell line.

4.2. Introduction

As already discussed in detail in the general introduction (Chapter 1), the activation and translocation of Bax to the OMM is a pivotal event within the intrinsic apoptotic pathway, and is implicated in the apoptotic cell death associated with several neurodegenerative disorders. Although gel filtration experiments suggest cytosolic Bax to be monomeric (Valentijn *et al.*, 2003), an emerging hypothesis is that Bax may interact with accessory proteins, both under resting conditions as well as during apoptosis where they have been proposed to function as cytosolic retention factors and facilitators of the Bax structure modifying and translocation events, respectively (Lucken-Ardjomande and Martinou, 2005).

As was described in the general introduction (Chapter 1), the translocation of Bax to the OMM is thought to rely on a discrete membrane-targeting domain within the C-terminus (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000; Schinzel *et al.*, 2004b). However, due to its hydrophobicity, this domain is normally internalised within the Bax protein structure in a hydrophobic pocket, only becoming accessible to the cytosolic environment during apoptosis when its membrane-targeting capability is required (Suzuki *et al.*, 2000). Chaperone proteins may therefore make an important contribution to the functional maturity of Bax during apoptosis by regulating the

exposure of the C-terminal tail and maintaining this hydrophobic region in a competent orientation for membrane targeting.

Chaperone proteins may also function to maintain Bax as an inactive cytosolic protein in the absence of cell stress. Such proteins would exert a critical regulatory hold over the response of Bax to apoptotic stimuli. However, by 2004, only one such cytosolic retention factor, humanin, had been identified in neuronal cells (Hashimoto *et al.*, 2001; Guo *et al.*, 2003). Studies involving other proposed Bax retentive factors had used different cell lines, for instance the molecular chaperone Hsp60 was identified as a Bax-binding protein in cardiac myocytes (Kirchhoff *et al.*, 2002), and the scaffold protein 14-3-3σ was shown to perform a similar role in epithelial cells (Nomura *et al.*, 2003). Although a comprehensive study of Bax-binding proteins in all tissues has yet to be accomplished, it could be that Bax is regulated by a different subset of proteins according to cell type. Hence, in this chapter, a proteomics-based search for Bax-binding proteins in a neuronal cell line will be described. This chapter will also present results obtained from a more focussed search for proteins binding specifically to the putative Bax C-terminal membrane-targeting domain. The identification of such regulatory proteins could have far-reaching implications in the development of new therapeutics to limit the excessive apoptotic cell death associated with neurological disorders in which Bax activity plays a central role.

4.3. Materials & Methods

4.3.1 Preparation of SH-SY5Y whole cell lysates

Control (DMSO-vehicle) and apoptotic (500nM STS; 5h) SH-SY5Y whole cell lysates were prepared as described in general materials & methods (Chapter 2).

4.3.2 Bax immunoprecipitation

Bax was immunoprecipitated overnight at 4°C from control and apoptotic SH-SY5Y whole cell lysate (2-5mg total protein) with a pan-reactive polyclonal Bax antibody (Upstate Biotechnology). Immunoprecipitation was performed as described in general materials & methods (Chapter 2) except proteins were captured using protein G-agarose beads.

4.3.3. Proteomic analysis of Bax co-immunoprecipitants

An overview of the proteomics-based approach used to identify Bax-binding proteins is provided in Figure 4.1. The proteomic studies described in this chapter were performed in collaboration with the ACE proteomics group.

4.3.4. 2D-SDS PAGE

Bax co-precipitants were separated by 2-dimentional (2D) SDS-PAGE. The first dimension separated proteins according to their isolectric point (pI) and the second dimension according to molecular weight (Mr). First dimension separation was performed using linear pH 3-10 IPG (immobilised pH gradient) strips (Biorad) using a Biorad protean IEF (isoelectric focussing) cell. Samples were diluted in IEF sample buffer (7M Urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.8% ampholytes) to reach a final

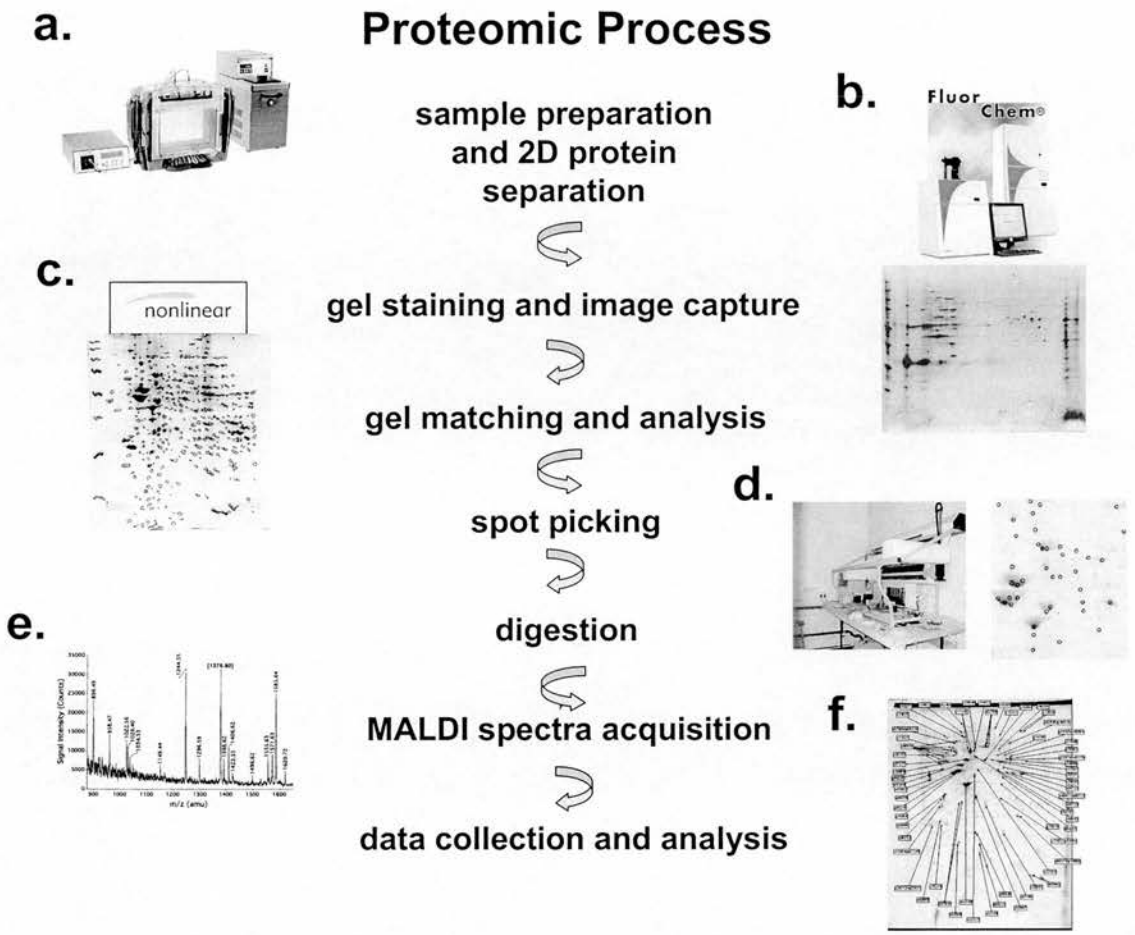


Figure 4.1. Proteomic analysis flow diagram of the proteomic analysis used in this thesis to identify Bax-binding proteins. Proteins for analysis were extracted form SH-SY5Y whole cell lysates and separated according molecular weight and isoelectric point by 2D gel SDS-PAGE **(a)**. Gels were then silver stained and imaged **(b)** for spot characterisation analysis by Phoretix 2D software **(c)**. Identified protein spots were picked by robot **(d)** and digested in trypsin for analysis of peptides by MALDI-TOF mass spectrometry **(e)**. Peptide mass fingerprints for each protein spot were compared to on-line proteomic databases and identities assigned to each protein spot **(f)**.

protein load of 300 μg (in 340μl) per strip. IPG strips were actively rehydrated for 12h then focussed at 500V for 1h, 1000V for 1h and 8000V for 32,000Vh. DTT (3.5%) was added to the cathode to maintain reducing conditions. Prior to running on the second dimension, IPG strips were equilibrated twice for 15min in 10ml of equilibration solution (0.1M tris HCl (pH 6.8), 5.5M urea, 0.3% glycerol, 0.035M SDS, and 0.065M DTT). For the second equilibration DTT was replaced by 14mM of iodoacetamide. The second dimension separation was performed using pre-cast plastic-backed continuous 12.5% polyacrylamide gels (Amersham Biosciences) at 1 Watt per gel for 18h using the manufacturer's proprietary buffer system. Gels were fixed for at least 1h in 40% methanol, 7% acetic acid and stained overnight using a mass spectrometry-compatible silver stain (Biorad).

4.3.5. Imaging and analysis

Five gels from each treatment group (vehicle- and STS-treated) were imaged using a FluorChem image analyser (AlphaInnotech) and gel images were stored as '.tif' files of 2.58 MB and 16-bit resolution. Gels were analysed using Phoretix 2D software (Non-linear Dynamics) by first allowing the software to outline each individual protein feature as a distinct area of stained gel. Protein features appearing across all five gels were identified as 'seed' matches. Where the Phoretix software had failed in spot definition, features were defined manually. The gels were then automatically matched, spot feature to spot feature, and an experiment created within the program to group vehicle- and staurosporine-treated gels.

4.3.6. Protein selection and identification

Selected protein features were extracted from the plastic-backed gels using a ProPic robot (Genomics Solutions). Plugs were collected into 96-deep-well plates (Abgene, Epsom, UK) and stored at -20°C in ethanol (20%). Protein in-gel trypsin digestion and MALDI (matrix-assisted laser desorption/ionization) target spotting were performed

using a Multiprobe II (PerkinElmer, Beaconsfield, UK) robotic system. Briefly, gel plugs were washed 3 times with 50 mM ammonium bicarbonate in 50% acetonitrile before being dehydrated with 100% acetonitrile, dried, rehydrated with unmodified trypsin (about 200 ng; Promega) and incubated overnight at 32°C. The digested protein mixture was spotted robotically onto a MALDI target with an equal volume of alpha-hydroxycinnamic acid in a solution of 50% acetonitrile with 0.1% trifluoroacetic acid (TFA).

4.3.7. Mass Spectrometry

Spectra were acquired automatically on a Voyager-DE STR mass spectrometer (Applied Biosystems, Warrington, UK) recording masses between 800 and 3500Da. The samples were calibrated internally using the trypsin autolysis peaks at 842.51Da and 2211.1046Da. Any spectra not automatically calibrated by this approach were re-calibrated manually before database interrogation. Peptide masses collected from mass spectra were submitted to search the SwissProt database (http://us.expasy.org/) through the Protein prospector website (http://prospector.ucsf.edu/) using Data Explorer and PS1 software (Applied Biosystems). Protein matches were assessed on the basis of the number of peptides matched to the target protein, the intensity of these peptides expressed as a percentage of all submitted peptides and MOWSE score.

4.3.8. Affinity Chromatography

Affinity chromatography columns were prepared using biotinylated peptides corresponding to the C-terminal 21 amino acids of wild type (biotin-TVTIFVAGVLTASLTIWKKMG) and S184V mutant (biotin-TVTIFVAGVLTA**V**LTIWKKMG) Bax (Albachem, Edinburgh, UK). Peptides were coupled to NeutrAvidin-agarose beads (Pierce, Cramlington, Northumberland, UK) according to the manufacturer's instructions and equilibrated in a mobile phase of 10mM PIPES, 150mM NaCl, 1% Tween pH6.5 using a FPLC system (GE Healthcare).

Affinity chromatography was performed using SH-SY5Y whole cell lysates (2-5mg total protein). Specifically bound proteins were eluted using 100mM glycine pH2 (1ml), separated by Tris/Glycine PAGE (Invitrogen) and visualised using a mass spectrometry-compatible silver stain. Protein bands were analysed by mass spectrometry as described above.

4.3.9. Co-immunprecipitation and Western blotting

Co-immunoprecipitation and Western blotting were performed as described in general materials & methods (Chapter 2). Briefly, Bax was immunoprecipitated from control and apoptotic SH-SY5Y whole cell lysates using a non-conformation-specific Bax antibody or a Bax antibody recognising the conformation-specific 6A7 epitope, respectively. Immunoprecipitated proteins were separated by SDS-PAGE and analysed by Western blot using appropriate antibodies.

4.4. Results

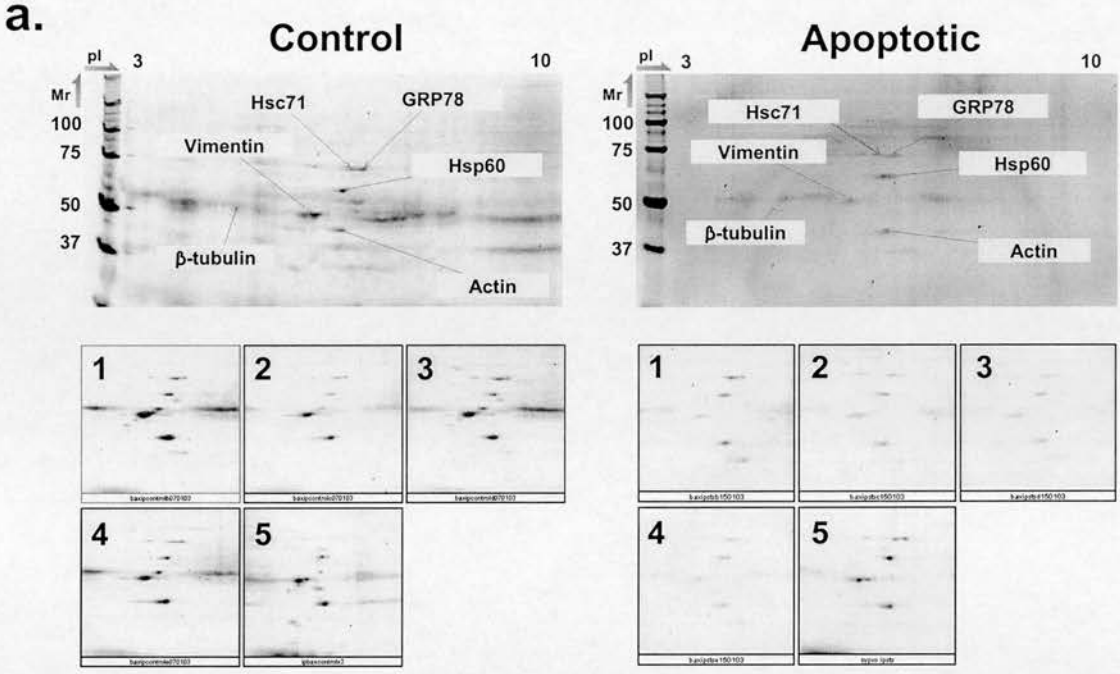
4.4.1 Endogenous Bax interacts with heatshock and cytoskeletal proteins

Soluble cytosolic Bax-binding proteins originating from control and apoptotic (STS;5h) SH-SY5Y cells were co-immunoprecipitated with Bax, analysed by 2D gel electrophoresis and identified by mass spectrometry. Although numerous spots were visible on the 2D gels, only six proteins were consistently present in the five replicate gels performed using both control and apoptotic cell lysates – see Figure 4.2. Identification of these Bax-binding factors by MALDI-TOF mass spectrometry revealed them to be: the heat shock proteins, Hsp60, Hsc71 and GRP78 and the cytoskeletal proteins, β-actin, tubulin and vimentin. Additional analysis of these interactions was performed using co-immunoprecipitation and Western blotting - see Figure 4.3. From these studies, interactions between Bax and Hsp60, β-actin and vimentin were confirmed, but not those between Bax and Hsc71, GRP78 or tubulin.

4.4.2 Hsp60, β-actin and nucleophosmin bind to the Bax C-terminal membrane-targeting domain

Further experiments were performed to identify proteins that may be involved specifically in regulating the translocation of Bax to the OMM. Because the hydrophobic C-terminal region of Bax has previously been implicated as a mitochondrial membrane targeting domain (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000), a synthetic peptide corresponding to the wild-type (WT) C-terminal 21 amino acids of Bax was used as bait to identify binding proteins in SH-SY5Y whole cell lysate by affinity chromatography. From this study, the cytoskeletal protein, β-actin, and the heat shock family proteins, Hsp60 and Hsp90, were identified as Bax C-terminal binding proteins – see Figure 4.4. However, when this experiment was repeated using a mutated (S184V) Bax C-terminal peptide, in addition to the three

a.



b.

Identified protein	Accession No.	Mr (kDa)	pI	MOWSE	Hits/submitted	Intensity (%)	Error
hsp60	P10809	61.1	5.7	6.4×10^3	7/70	20	7±9
hsc71	P11142	70.9	5.4	4.2×10^6	12/70	24	8 ± 13
GRP78/Bip	P11021	72.3	5.1	5.5×10^7	13/64	15	11 ± 10
vimentin	P08670	53.7	5.1	6.9×10^9	23/70	44	14 ± 18
tubulin	Q9BQE3	49.9	5.0	5.2×10^7	11/50	40	5 ± 7
β-actin	P60709	41.7	5.3	1.9×10^4	9/60	45	10 ± 10

Figure 4.2. 2-Dimensional (2D) gel analysis of Bax co-precipitating proteins in control and STS-treated SH-SY5Y cells. Bax co-precipitants in vehicle-treated **(Control)** and 5h STS-treated **(apoptotic)** SH-SY5Y whole cell lysates were analysed by 2D gel electrophoresis and identified by mass spectrometry **(a)**. The master gel (top) shown for each experiment is a composite of the five individual gels pictured below. Six proteins were consistently eluted with Bax under control and apoptotic conditions. The accompanying table **(b)** details, for each protein, the molecular mass in kDa (Mr), isoelectric point (pI), the molecular weight score (MOWSE), the number of peptides matched from the total of peptides submitted (hits/submitted), the overall intensity of the peptides matched as a percentage of the total peptide intensity (intensity) and the error between the predicted and measured peptide masses expressed as mean ± standard deviation (in parts per million, p.p.m; error).

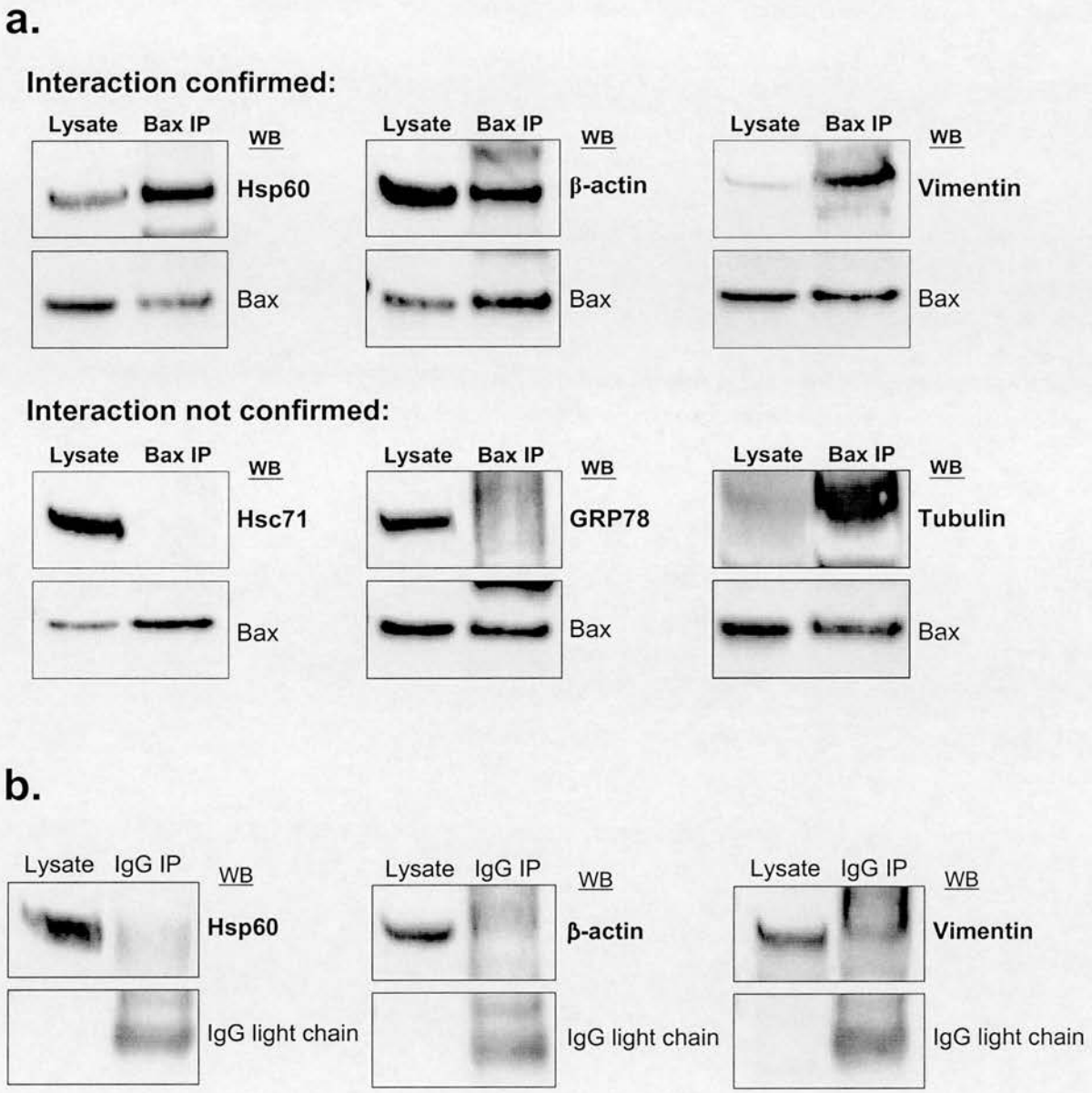


Figure 4.3. Confirming interaction between Bax and identified 2D binding proteins by co-immunoprecipitation and Western blot. Confirmation of interaction between Bax and Hsp60, β -actin and vimentin was achieved, whereas interaction between Bax and Hsc71, GRP78, and tubulin could not be confirmed using co-immunoprecipitation **(a)**. Specificity of interaction between Bax and Hsp60, β-actin and vimentin was tested by immunoprecipitating lysate with rabbit IgG **(b)**. Detection of proteins by Western blot (WB) in whole cell lysate (lysate) and Bax immunoprecipitate (Bax IP) are as indicated. Images shown are representative of at least three independent experiments.

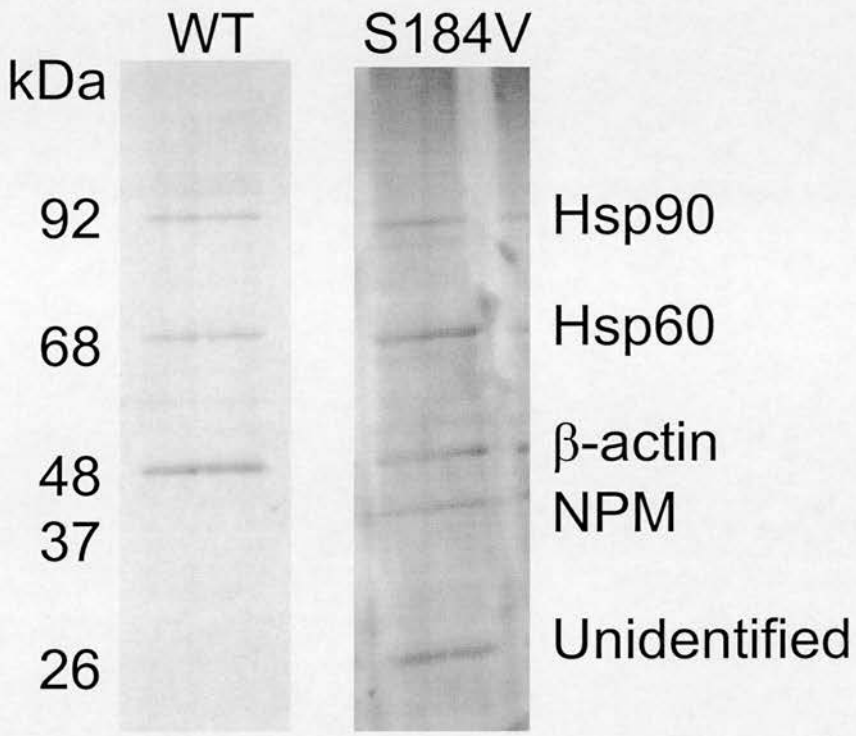


Figure 4.4. Identification of Bax C-terminus-binding proteins by affinity chromatography. Proteins of molecular weight 48, 68 and 92kDa were eluted with both the wild type (WT) and mutant (S184V) Bax C-term peptides, and were subsequently identified by mass spectrometry as β-actin, Hsp60 and Hsp90, respectively. However, only two proteins of molecular weight 26 and 37kDa bound specifically to the mutant peptide. The 37kDa protein was subsequently identified as nucleophosmin by mass spectrometry; however the identity of the 26kDa band could not be established. The image shown is representative of three independent experiments.

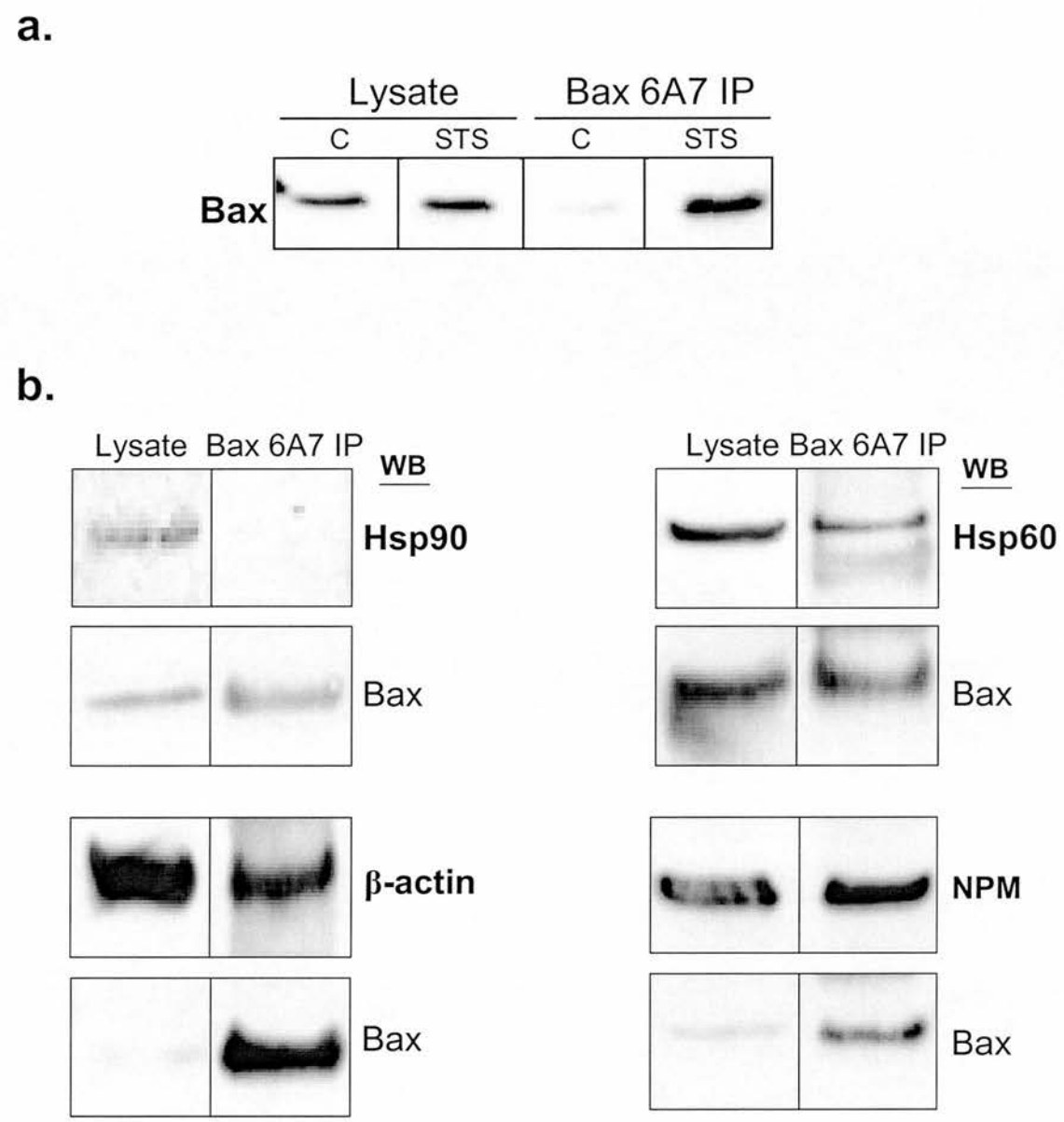


Figure 4.5. Confirming interaction between Bax and identified Bax C-terminal-binding proteins by co-immunoprecipitation and Western blotting. The presence of activated Bax in STS-treated lysates was confirmed by immunoprecipitating Bax with the conformation-specific antibody 6A7 **(a)**. Co-immunoprecipitation studies confirmed the interaction between 6A7-reactive Bax and Hsp60, β-actin and nucleophosmin (NPM) in apoptotic cell lysate, but not that between Bax and Hsp90 **(b)**. Detection of proteins by Western blot (WB) in whole cell lysate (lysate) and immunoprecipitate (Bax 6A7 IP) are as indicated. Images shown are representative of at least three independent experiments.

proteins identified using the wild type peptide, two further bands of approximately 26kDa and 42kDa were present in the final elution – see Figure 4.4. The 42kDa protein was subsequently identified by mass spectrometry as the nucleolar phosphoprotein, nucleophosmin, whereas no identity could be assigned to the 26kDa species.

Since all four identified proteins bound to the mutant peptide representing activated Bax, co-immunoprecipitation studies to confirm their interaction with Bax were performed using apoptotic (STS-treated) SH-SY5Y cell lysates. The presence of activated Bax in this lysate was confirmed by immunoprecipitating Bax with an antibody recognising the active conformation-exposed 6A7 epitope - see Figure 4.5a. Co-immunoprecipitation experiments performed using this antibody confirmed the interaction between activated Bax and Hsp60, β-actin and nucleophosmin, but not that between activated Bax and Hsp90, under apoptotic conditions – see Figure 4.5b.

4.5. Discussion

This chapter addressed two aims: 1) to identify proteins interacting with quiescent Bax under normal conditions in neuronal cells and 2), to identify proteins binding specifically to the putative transmembrane domain located within the Bax C-terminal tail. Proteins belonging to the first category are candidate cytosolic retention factors, functioning to protect the cellular environment from premature activation of Bax. The latter proteins, however, due to their specific binding to the Bax membrane-targeting domain, are of special interest as they are potentially important regulators of the translocation of Bax to the mitochondrial surface. The potential contribution that each of these proteins may bring to the regulation of Bax bioactivity will now be discussed.

In recent years, compelling evidence has accrued suggesting that Bax does not reside, nor function, in the cytosol in isolation (reviewed by Lucken-Arjomande & Martinou, 2005). In this chapter, several novel Bax-interacting proteins, originating primarily from the heat shock protein (Hsp) family and the cytoskeleton, were identified.

During episodes of cellular stress, Hsp proteins protect the cellular environment, in part, by suppressing the activation and progression of the intrinsic apoptotic pathway (Takayama *et al.*, 2003). Certain Hsp family members are known to inhibit the activity of pro-apoptotic Bcl-2 family proteins, including Bax (Kirchhoff *et al.*, 2002; Mao *et al.*, 2004; Gotoh *et al.*, 2004) Bak (Kirchhoff *et al.*, 2002) and Bad (Shan *et al.*, 2003), sequester cytochrome c upon its release from mitochondria (Bruey *et al.*, 2000) and disrupt formation of the apoptosome (Beere *et al.*, 2000; Saleh *et al.*, 2000).

The identification of Hsp60 as a Bax-binding protein in this thesis confirmed an earlier observation made by Kirchoff and colleagues who first demonstrated this interaction in hypoxic cardiac myocytes in 2002 (Kirchhoff *et al.*, 2002). Hsp60 has also been reported to interact with Bak on the mitochondrial surface (Kirchhoff *et al.*, 2002) and

attenuate the apoptogenic activity of the BH3-only protein, Bad, in cardiac myocytes exposed to doxorubicin (Shan *et al.*, 2003). Thus, at least within cardiac cells, Hsp60 appears to inhibit apoptosis by down regulating the apoptogenic function of multiple pro-apoptotic Bcl-2 family proteins.

Hsp60 is a molecular chaperone primarily localised to the matrix of the mitochondria where it refolds key mitochondrial proteins (Ostermann *et al.*, 1989). However, a small proportion (around 15-20%) is also present in the cytosol (Itoh *et al.*, 1995; Mills *et al.*, 1999). Cytosolic Hsp60 contains an N-terminal signal sequence that directs the targeting of Hsp60 to the mitochondria in response to cellular stress (Itoh *et al.*, 2002). The translocation of Hsp60 to the plasma membrane has also been observed under such conditions (Gupta and Knowlton, 2002).

The identification of Hsp60 as a Bax-binding factor in cardiac myocytes provided the first evidence supporting a direct role for Hsp60 in regulating stress-induced apoptotic pathways (Kirchhoff *et al.*, 2002). From their observations, Kirchhoff and colleagues concluded that Hsp60 retained Bax in the cytosol under normal conditions and that dissociation between these two proteins was required for Bax to complete its translocation to the OMM. The identification of Hsp60 as a Bax binding partner in the SH-SY5Y neuroblastoma cell line suggests that Hsp60 may perform a similar role in cells of neuronal origin.

However, the first reported involvement of Hsp60 in regulating apoptotic mediators came in 1999 when two groups recognised its role in accelerating the maturation of caspase-3 within the mitochondrial matrix (Xanthoudakis *et al.*, 1999; Samali *et al.*, 1999). This led researchers to believe that Hsp60 could also function as a death-promoting protein, perhaps under certain physiological circumstances. However, within the mitochondria, Hsp60 functions as an important chaperone factor to numerous matrix proteins (Ostermann *et al.*, 1989). Since a population of procasapse-3 is also found within the mitochondria (Mancini *et al.*, 1998) it is probable that Hsp60 assists in the

conformational maturation of procaspase-3 as it would with any other client protein. Thus, the observed involvement of Hsp60 in caspase-3 maturation does not necessarily mean that Hsp60 can also function as a pro-apoptotic protein.

Nevertheless, the affinity chromatography studies performed in this thesis identified Hsp60 as one of five proteins binding to the Bax C-terminal transmembrane domain, implicating Hsp60 as a potential Bax chaperone during its translocation to the OMM. The α9 helix of Bax, which forms part of the C-terminal transmembrane domain, is hydrophobic and is therefore internalised within the Bax molecule in a binding pocket (Suzuki *et al.*, 2000). However, the transmembrane domain is not exclusively hydrophobic as it also contains several hydrophilic polar residues (Thr172, Thr174, Thr182, Ser184 and Thr186) that, apart from Ser184, form a cytosolic face that is thought to greatly improve the solubility of the Bax molecule within the cytosol (Suzuki *et al.*, 2000). Consequently, a portion of the Bax C-terminal tail is accessible to binding prior to its activation and translocation. Thus, instead of acting as a translocation factor, Hsp60 could instead associate with the cytosolic surfaces of the Bax C-terminus under normal conditions as a means of maintaining Bax in an inactive conformation. This hypothesis is in agreement with previous findings illustrating the inhibitory role of Hsp60 on Bax bioactivity (Kirchhoff *et al.*, 2002; Knowlton and Gupta, 2003; Shan *et al.*, 2003) as well as accompanying data presented in this thesis demonstrating Hsp60 as a protein binding endogenous Bax under normal conditions.

Heat shock cognate protein 71 (Hsc71) and the ER-localised 78kDa glucose-regulated protein, GRP78, both members of the Hsp70 protein family, were initially identified as Bax co-precipitants in the 2D gel proteomic study but could not be confirmed as Bax-interacting proteins by co-immunoprecipitation. This discrepancy may have arisen as a result of using a different antibody to immunoprecipitate Bax in both experiments. Alternatively, the transient, low binding affinity commonly observed between chaperones and their substrates (Hendrick and Hartl, 1993) may make it difficult to

investigate this class of protein-protein interaction using co-immunoprecipitation-based approaches.

Nevertheless, that both proteins were identified from the 2D gel proteomic study as proteins interacting with Bax under resting conditions is in agreement with their known protective cellular roles as heat shock proteins. Hsp71 is a constitutively expressed protein (Ingolia and Craig, 1982) and, as a chaperone, is involved in folding and facilitating transportation of nascent polypeptides across intracellular membranes (Tavaria *et al.*, 1995). A specific anti-apoptotic role has not been attributed to Hsc71 *per se*, but its stress-induced variant, Hsp70, is an established cytoprotective protein that has been implicated in inhibiting key molecular events within the intrinsic apoptotic pathway, such as stress-induced JNK signalling (Mosser *et al.*, 1997; Gabai *et al.*, 1997), cytochrome c release (Mosser *et al.*, 2000) and apoptosome formation (Beere *et al.*, 2000; Saleh *et al.*, 2000). As Hsc71 is a constitutively expressed protein, it could instead function as a cytoprotective protein under resting cellular conditions, for instance as a Bax retentive protein. GRP78 (also known as immunoglobulin binding protein Bip) promotes the folding of unfolded proteins that have accumulated within the ER lumen (Hendershot *et al.*, 1994) and is an integral component of the unfolded protein response (UPR) (Lee, 2001). Dysfunction of the ER stress-induced UPR is thought to be a major contributor to neuronal cell death in Alzheimer's disease and Parkinson's disease (Katayama *et al.*, 1999; Imai *et al.*, 2001; Sato *et al.*, 2001). In one study, expression levels of GRP78 were observed to be significantly reduced in the brains of Alzheimer's disease patients (Katayama *et al.*, 1999) and, in the same study, over-expressing GRP78 in a neuroblastoma cell line containing a presenilin-1 (PS1) mutation completely restored resistance to ER stress-induced apoptosis. A direct involvement of GRP78 in regulating ER stress-induced apoptotic pathways has been previously demonstrated. In non-stressed cells, GRP78 binds to and maintains inactive the key ER stress transducing protein, ATF6 (Lee, 2005) and the ER-associated caspases, caspase-7 and -12 (Rao *et al.*, 2002). Only upon dissociation from GRP78 are these proteins able to initiate downstream apoptotic pathways (Lee, 2005). A hypothetical interaction between Bax

and GRP78 is possible, as GRP78, although predominantly localised within the ER lumen, has also been identified within the cytosol, particularly during cell stress (Rao *et al.*, 2002). Furthermore, an apoptogenic role for Bax at the ER is becoming increasingly evident, where it has been proposed to initiate an apoptosome-independent route to caspase-activation via caspase-12 (Zong *et al.*, 2003). Further studies will be required in order to evaluate whether a functional interaction between Bax and GRP78 has implications in protecting neurones against ER stress-induced apoptosis.

Three proteins, β-actin, tubulin and vimentin, representatives from each of the three main cytoskeletal structures in the cell, were also identified as proteins binding to Bax under normal cellular conditions.

The cytoskeletal protein, tubulin, although seen in the 2D gel proteomic study, could not be confirmed as a Bax-interacting protein by co-immunoprecipitation and Western blotting. Tubulin is expressed at low levels in undifferentiated SH-SY5Y cells (Butler *et al.*, 2001) and this may have hampered our ability to detect an interaction between tubulin and Bax using co-immunoprecipitation. However, a recent study has demonstrated a direct interaction between recombinant Bax and extracted rat brain tubulin (Knipling and Wolff, 2006), although it remains to be confirmed whether a functionally relevant interaction occurs between endogenous proteins.

β-actin and vimentin, originating from the microfilament and intermediate filament components of the cytoskeleton, respectively, were identified and confirmed from these proteomic studies as Bax-interacting proteins. Interaction between pro-apoptotic Bcl-2 family proteins and cytoskeletal proteins is not unprecedented. The BH3-only proteins, Bim and Bmf, are known to associate with dynein motor components of the cytoskeleton as a means of sensing and responding to intracellular damage (Puthalakath *et al.*, 1999; Puthalakath *et al.*, 2001). However, direct interactions between Bax and β-actin or vimentin have not been identified previously.

Vimentin is one of the most ubiquitous intermediate filament proteins in mammals where it forms a cytoplasmic scaffold to stabilise the cellular architecture. Aside from this structural role, vimentin has been hypothesised to conduct signal transduction pathways as a substrate for numerous protein kinases, including the apoptosis-suppressing kinases, Raf-1 and PKC (Eriksson *et al.*, 2004). Phosphorylated vimentin is also known to sequester the 14-3-3 protein (Tzivion *et al.*, 2000), an important regulator of apoptosis and cell-cycle progression through its ability to orchestrate the subcellular localisation of numerous proteins (Hermeking, 2003). Of the seven human 14-3-3 isoforms, 14-3-3σ has been attributed with the greatest tumour-suppressor activity, and has been proposed to be a cytoplasmic anchor of Bax (Samuel *et al.*, 2001). Recently it has been demonstrated that vimentin interacts with 14-3-3β which in turn is known to sequester the phosphorylated form of the pro-apoptotic BH3-only protein, Bad (Schietke *et al.*, 2006). Thus, whether vimentin binds Bax directly, or through an intermediate such as a 14-3-3 isoform, its influence on Bax activity is likely to be that of a cytosolic retentive factor.

β-actin was identified as a protein interacting with the whole Bax molecule as well as with the Bax C-terminal transmembrane domain. The latter finding suggests that, under cellular duress, Bax may utilise components of the cytoskeleton as a means of translocation to the mitochondrial surface. This hypothesis is supported by a recent study demonstrating the accumulation of β-actin at the mitochondrial surface prior to the translocation and insertion of Bax during exposure of various cell lines to STS and UV-irradiation (Tang *et al.*, 2006). The authors of this report proposed that the actin cytoskeleton might therefore facilitate the mitochondrial recruitment of Bax and other pro-apoptotic proteins from the cytosol during apoptosis. This study, however, did not attempt to demonstrate whether a direct interaction existed between actin and Bax in these cells. Nevertheless, a functioning actin cytoskeleton has been shown to be essential for the transduction of a death signal to mitochondria during TNFα treatment in L929 cells (Li *et al.*, 2004a). Furthermore, the actin cytoskeleton presents a natural conduit for transporting pro-apoptotic proteins to the mitochondria, as the migration of

mitochondria along Arp2/3-generated F-actin filaments has been observed, at least in yeast (Boldogh *et al.*, 2001). Moreover, the characteristic perinuclear clustering of mitochondria observed during TNFα-induced apoptosis (De Vos *et al.*, 1998) is, in addition to microtubules, mediated by association with actin microfilaments (Li *et al.*, 2004a). Disruption to the dynamic state of actin has been linked to apoptosis initiated by the mitochondrial release of reactive oxygen species (Posey and Bierer, 1999; Gourlay *et al.*, 2004) and several cytoskeletal components also contribute to the execution phase of apoptosis where caspase-cleaved truncated actin (tActin) and vimentin (tVimentin) fragments are both able to induce morphological changes associated with apoptosis when ectopically expressed in culture (Mashima *et al.*, 1999; Byun *et al.*, 2001). It is therefore feasible that the actin cytoskeleton, through its association with Bax, may play an important role in facilitating the transduction of a stress-induced apoptotic signal to the mitochondrial surface.

In addition to Hsp60 and β-actin, affinity chromatography studies using Bax C-terminal peptides (wild-type and mutant S184V) identified three other proteins with molecular weights of 26kDa, 37kDa and 90kDa. The 37kDa and 90kDa proteins were subsequently identified using mass spectrometry as nucleophosmin and Hsp90, respectively. No identity, however, could be assigned to the low abundance 26kDa protein, despite repeated attempts. Interestingly, the isolation of an unknown 25-26kDa component of a Bax complex using gel exclusion chromatography was reported by another laboratory at the fifth Interdisciplinary Euroconference on Mechanisms of Cell Death and Disease in Portugal in 2003. However, these data have not yet been published. Subsequent co-immunoprecipitation analyses failed to confirm the interaction between Bax and Hsp90. Although Hsp90 is a reportedly highly abundant protein in most cell types (Lai *et al.*, 1984), its observed expression level in cultured SH-SY5Y cells was low, which may have impacted on the ability to confirm the Bax-Hsp90 interaction by co-immunoprecipitation. Furthermore, Hsp90 has been shown to associate with a large number of co-chaperones (Picard, 2002) and it is therefore

possible that Hsp90 may have been identified as one component of such a complex that does not interact directly with Bax.

Significantly, the molecular chaperone nucleophosmin (NPM; also known as B23 or numatrin) was observed to bind exclusively to the mutant peptide and therefore appears to specifically recognise the activated Bax conformation. Nucleophosmin had recently been proposed as a nuclear factor promoting the transcriptional activity and stability of the tumour suppressor protein, p53, during genotoxic stress (Colombo *et al.*, 2002). Thus, the identification of nucleophosmin as a novel Bax-binding protein and a potential chaperone to Bax during its translocation to the OMM provides evidence in support of an additional regulatory mechanism for this protein within the intrinsic apoptotic pathway.

4.6. Summary

The results presented within this chapter demonstrate that quiescent Bax, extracted from SH-SY5Y whole cell lysates, is found in association with several regulatory factors, including the heat shock family protein, Hsp60, and the cytoskeletal proteins, β-actin and vimentin. Furthermore, Hsp60 and β-actin can bind specifically to the Bax C-terminal tail, a region thought to be implicitly involved in targeting Bax to the mitochondrial surface. These proteins may function either as putative retentive factors or facilitators of Bax translocation, depending on whether or not the cell is undergoing apoptosis. Several additional proteins were isolated in the same study as the Bax co-precipitants, including tubulin, Hsc71 and GRP78. Although each of these may make a significant contribution to Bax function, subsequent analyses of their relationship with Bax by co-immunoprecipitation and Western blotting, in this study, could not conclusively demonstrate the existence of a direct interaction. Finally, nucleophosmin, a molecular chaperone that has previously been linked with promoting p53 activity, was isolated from SH-SY5Y whole cell lysates as a protein binding specifically to a mutant (S184V) Bax C-terminal peptide. This novel interaction implicates nucleophosmin as a

potential regulator of the mitochondrial targeting mechanism of Bax – an event that is essential for Bax to induce cell death but which, despite extensive research, remains poorly understood. Determining the structural and functional relationships between Bax and nucleophosmin will therefore form the basis of the following two chapters (5 & 6), respectively.

CHAPTER 5: Characterising the molecular interaction between Bax and nucleophosmin

5.1. Chapter aims

Nucleophosmin was identified as a Bax-binding protein by affinity chromatography as described in chapter 4. In order to investigate the interaction between Bax and nucleophosmin in more detail, the aims of this chapter are: 1) to express recombinant full-length human Bax and nucleophosmin recombinant proteins, and 2) to develop a cell-free amplified luminescent proximity assay in which to characterise the molecular interaction between these recombinant proteins.

5.2. Introduction

5.2.1. Recombinant protein synthesis

The production of recombinant proteins from a wide variety of expression systems is a frequently used approach to investigate protein structure, function and protein-protein interactions. The quantity of protein required depends on the application: For screening purposes, a relatively small amount (micrograms or less) of a large number of proteins is sufficient, whereas for certain therapeutic applications (eg antibody production) quantities approaching the metric ton scale are needed (Andersen and Krummen, 2002).

The choice of expression system depends on the amount of protein required as well as the intended use. In the laboratory, recently developed cell-free, *in vitro* expression systems using mammalian or *E.coli* cell extracts can provide a rapid and convenient approach to synthesising small quantities of proteins for use in high throughput screening assays (Swartz, 2001). Cell-free expression systems also have the added benefit of being able to express highly toxic proteins, such as Bax. Larger protein yields are obtainable from mammalian cell expression systems (Chinese hamster ovary (CHO)

being the most commonly used) that have the added advantage of providing all the necessary translational machinery to ensure correct folding and post-translational modification of expressed nascent proteins. However, the most common expression system used in the lab is *Escherichia coli* due to its relative simplicity, inexpensive and fast high-density cultivation, its well-characterised genetics and the large number of compatible tools available within modern biotechnology (Swartz, 2001; Sorensen and Mortensen, 2005). Furthermore, recent advances in the genetic modification of *E.coli* hosts have greatly improved their ability to express recombinant proteins that are correctly folded and have the bioactivity associated with endogenous proteins. For example, modifications that encourage the expression of correctly folded proteins include *E.coli* mutants that co-express chaperone proteins (particularly ribosomal-associated trigger factor, GroEL and DnaK chaperones) (Nishihara *et al.*, 2000; Ikura *et al.*, 2002) or enhance disulphide bond formation (Bessette *et al.*, 1999).

Expression of recombinant proteins is normally induced from a plasmid DNA vector that is compatible with the transcriptional/translational machinery of the host cell. Genetic elements typically found within most expression plasmids (as recently reviewed by Sorensen and Mortensen, 2005) include the origin of replicon (*ori*), which regulates plasmid copy number, an antibiotic resistance marker (commonly against Ampicillin, Kanamycin or Chloramphenicol), transcriptional promoters (eg, bacteriophage T7 late or human cytomegalovirus (CMV) immediate early promoters), translational initiation regions (TIRs), which contain ribosome-recognition sequences specific to either eukaryotic or prokaryotic hosts, and transcriptional and translational terminators, such as poly-Adenosine (Poly-A) tails.

To ensure compatibility with the *E.coli* translational machinery, transcribed messenger RNA (mRNA) must have a ribosome binding site (RBS) containing a Shine-Dalgarno sequence (UAAGGAGG) 5-9 nucleotides upstream from an initiating methionine-encoding (AUG) start codon (Ringquist *et al.*, 1992). The RBS is recognised by the 16S component of the ribosome which then initiates translation in prokaryotic systems.

Eukaryotic translational machinery also uses AUG as the start codon, but the initiation of translation in this case requires a Kozak consensus sequence (CCA/GCC**AUG**G) that includes several nucleotides upstream and a single nucleotide downstream of the proximal methionine (Kozak, 1987). Translation in eukaryotic and prokaryotic hosts is terminated when the ribosome/tRNA complex reaches a stop codon at the end of the mRNA transcript. UAA is preferred by *E.coli*, but UAG and UGA are also used (Crick, 1966; Poole *et al.*, 1995)

High level gene expression from a plasmid vector within the host cell requires a strong promoter. The bacteriophage T7 promoter forms the basis of the widely used pET expression system, developed by Studier and colleagues and subsequently commercialised by Novagen, for the high yield production of recombinant proteins in *E.coli*. The T7 promoter is not recognised by *E.coli* RNA polymerase but is instead recognised by the λDE3 phage fragment-encoded T7 RNA polymerase, which is incorporated into the host cell genome (Studier *et al.*, 1990; Dubendorff and Studier, 1991). Expression of T7 RNA polymerase is under the control of the lactose analogue isopropyl-β-D-1-thiogalactopyranoside (IPTG)-inducible *lac*UV5 promoter (Baneyx, 1999). Thus, expression of the recombinant protein can be induced by the addition of IPTG (typically 50-100μM) to the *E.coli* culture. However, low-level basal or "leaky" expression of T7 RNA polymerase in the absence of IPTG can lead to plasmid expression instability, which is of particular concern if the recombinant protein is toxic to *E.coli*. Leaky expression of a toxic recombinant protein can lead to the selection of non-expressing cells over transformants within a culture prior to induction. A widely used strategy to overcome this is to use host cells expressing basal levels of the T7 RNA polymerase inhibitor, T7 lysozyme, from pLysS or pLysE plasmids (Studier, 1991). Additionally, expression of the *lac* repressor, lacI, inhibits induction of T7 RNA polymerase expression from the *lac*UV5 promoter, and incorporation of the *lacI* gene, together with a *lac* operator downstream from the T7 promoter on the expression plasmid, inhibits transcription of the target gene by T7 RNA polymerase. Transcription is initiated when IPTG binds and triggers the release of lacI from the *lac* operator. An

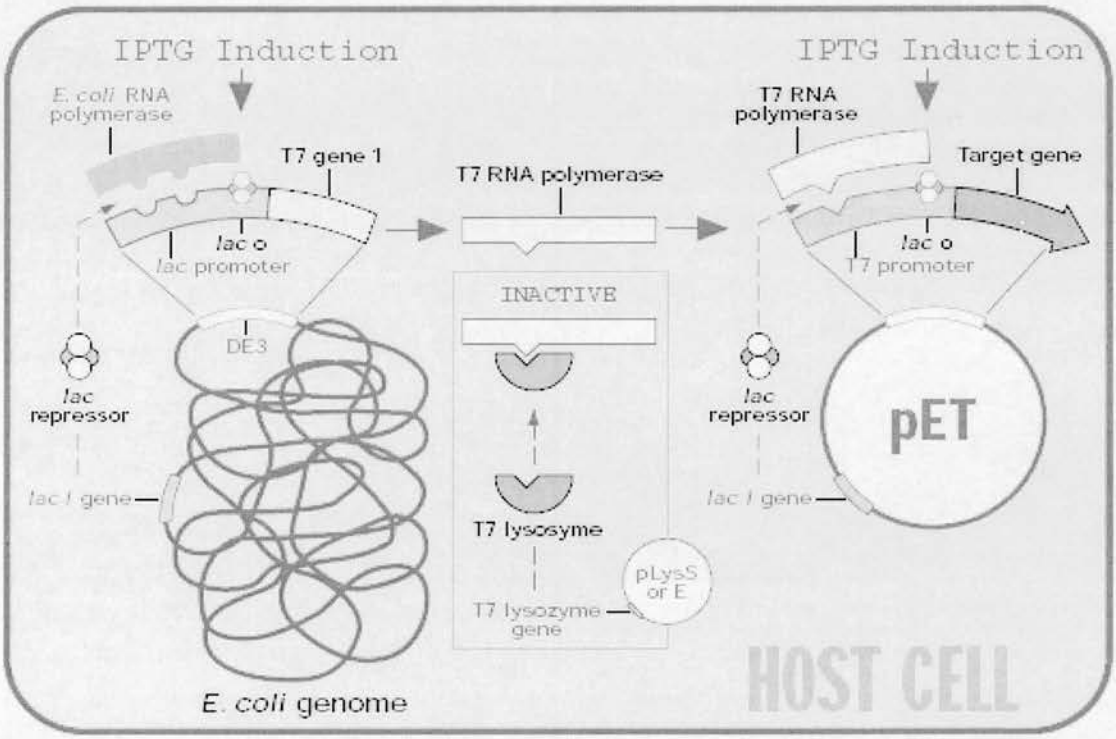


Figure 5.1. Control elements within the pET expression system. Basal transcription of T7 RNA polymerase in the uninduced state can lead to the negative selection of transformants within the *E.coli* population. Two genetically-regulated strategies have been developed to counteract this. Hosts carrying either pLysS or pLysE plasmids express T7 lysozyme, which is a natural inhibitor of T7 RNA polymerase. pLysS expresses a small amount of T7 lysozyme, whereas pLysE hosts produce more enzyme which allows for more stringent control. Expression of the lac repressor from *lacI* genes encoded in both the host genome and the pET plasmid provides additional control of transcription by inhibiting the binding of *E.coli* and T7 RNA polymerases to the *lac*UV5 and bacteriophage T7 promoters, respectively. Addition of IPTG to the *E.coli* culture disociates lacI from the *lac* operator, induces transcription of T7 RNA polymerase from the *lac*UV5 promoter which then induces transcription of the target gene from the pET plasmid. Image taken from Novagen *in*Novations Newsletter (1994). Vol. 1., No. 1.

overview of such control elements within the pET expression system is depicted in Figure 5.1.

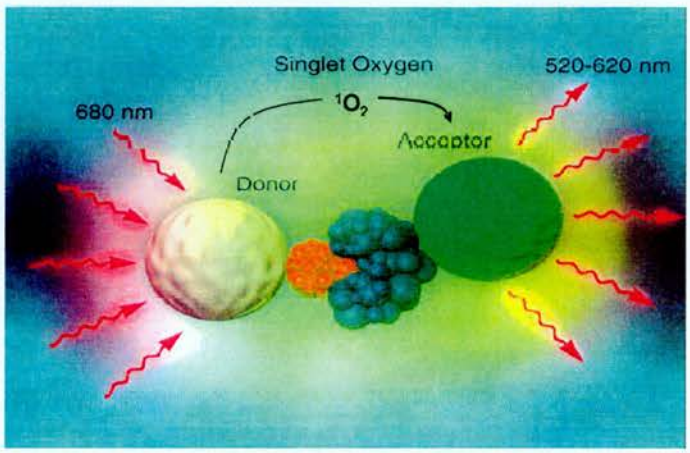
The versatility of recombinant protein expression is illustrated by the ability to attach fusion partners, or "tags", to either the N- or C-termini of the nascent protein in order to improve solubility (Davis *et al.*, 1999), protein yield (Arechaga *et al.*, 2003) or to ensure compatibility with downstream affinity-based purification methods (Terpe, 2003). Two commonly used tags to accommodate affinity-based purification of recombinant proteins are the polyhistadine(His) tag, which is compatible with immobilized metal affinity chromatography (IMAC), and the glutathione S-transferase (GST) tag, for purification using glutathione-based resins (Sorensen and Mortensen, 2005).

5.2.2. Principles of the cell-free amplified luminescence proximity assay

The analysis of protein-protein interactions using recombinant proteins within a cell-free setting provides several advantages over analyses performed *in situ*. Firstly, recombinant expression systems are capable of producing quantities of a specific protein often unattainable from natural host cells. Secondly, the truly homogeneous environment of an *in vitro* system permits the researcher to determine whether or not a direct interaction between two proteins exists. Thirdly a robust biochemical and pharmacological analysis of the interaction between two proteins can be performed. For example, dose-response experiments can determine interaction stoichiometry, competition assays can provide an assessment of relative binding affinities, and the inclusion of specific inhibitors can lead to the identification of binding interfaces (domains or motifs) within protein structures. Overall, the use of an *in vitro* binding assay provides an effective way to corroborate data obtained from experiments performed using endogenous proteins.

For the purpose of investigating the interaction between recombinant Bax and nucleophosmin in this thesis, two *in vitro*-based interaction assay technologies were

a.



b.

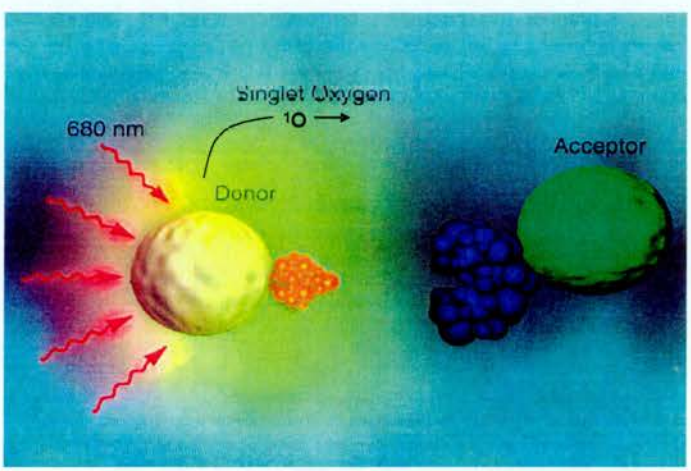


Figure 5.2. Principle of the AlphaScreen proximity assay. When biological interactions between conjugated proteins bring the Donor and Acceptor beads into close proximity **(a)**, singlet oxygen ($^1O_2$) released from laser-excited Donor beads stimulates fluorophores on Acceptor beads to emit a highly amplified luminescent signal of 520-620 nm. However, in the absence of a biological interaction **(b)**, Donor and Acceptor beads are separated by more than 200nm and consequently the emitted singlet oxygen decays and no amplified signal is generated. Images modified from Principles of AlphaScreen data sheet (PerkinElmer Inc.).

considered: an amplified luminescent proximity homogeneous assay (AlphaScreen; PerkinElmer) and a biomolecular binding platform based on surface plasmon resonance (SPR) technology (Biacore Life Sciences). Although each approach has its own specific strengths, the AlphaScreen system was chosen due its ease-of-use, fast reading times and high sensitivity (Glickman *et al.*, 2002).

The principle of luminescent oxygen channelling, which forms the basis of AlphaScreen technology, was first described in 1994 (Ullman *et al.*, 1994) and has since been commercialised by PerkinElmer Inc. Using this technology, the two proteins of interest are conjugated to separate hydrogel-coated polystyrene microbeads (donor and acceptor beads) and incubated together for up to several hours under ambient conditions. If a biological interaction between the proteins takes place, the acceptor and donor beads are brought together in close proximity.

Upon excitation of the donor beads with a high energy laser of wavelength 680nm, ambient oxygen is converted to an excited singlet state ($O_2 \rightarrow {}^1\Delta_g O_2$) which then diffuses out into the surrounding aqueous environment. However, singlet oxygen has a short lifetime in aqueous solutions (4μs) which therefore limits its diffusion from the donor beads to a distance of approximately 200nm. Only if a biological interaction brings the two proteins into close proximity are singlet oxygen molecules able to diffuse across from the donor beads to the acceptor beads. On the surface of the acceptor beads, the singlet oxygen reacts with thioxene derivatives to generate chemiluminescence at 370nm. This further stimulates fluorophores on the acceptor beads to emit a luminescent signal at 520-620nm (Ullman *et al.*, 1994) – see Figure 5.2a. However, if no biological interaction exists between the proteins, then the acceptor beads will be out-of-range of the emitted singlet oxygen molecules and thus only a background signal will be detected – see Figure 5.2b.

The AlphaScreen technology is highly sensitive – a photosensitiser on the Donor beads generates up to 60,000 singlet oxygen molecules per bead – which means only very

small volumes (< 5µl) of reagent and protein are required to generate a detectable signal. Consequently, multiple experiments can be performed simultaneously in 384-well plates, facilitating the provision of HTS capability, if required. The hydrogel-coated beads of uniform size minimize non-specific binding, and the high excitation wavelength of 680nm makes it unlikely that the presence of biological or assay substances in the reaction mix will interfere. The most likely source of error in this system is therefore attributed to the operator. AlphaScreen technology is designed for automated HTS, but manual operation is possible. However, due to the tiny reagent volumes involved and the light-sensitive nature of the fluorophore-coated beads, a meticulous and precise approach to each experiment performed using this assay is critical if accurate and consistent data are to be obtained.

5.3. Materials & Methods

5.3.1 Chemicals

All restriction enzymes were obtained from Promega. All plasmid DNA vectors were supplied by Invitrogen, unless otherwise stated.

5.3.2 Subcloning of Bax and Nucleophosmin cDNAs into expression vectors

Full length open reading frame (ORF) clones for human Bax and nucleophosmin were obtained from Invitrogen (Ultimate™ human ORF clones; ID numbers IOH130038 and IOH7470, respectively) and supplied in the Gateway® entry vector pENTR™221. Plasmid DNA was transformed into competent DH10B T1-Phage resistant *E. coli* cells (Invitrogen). Transformed colonies were selected, propagated and plasmid purified (Wizard Plus SV minipreps; Promega) using standard molecular procedures (Sambrook & Russel, 2001).

To enable *in vitro* expression of eukaryotic proteins, Bax and nucleophosmin full-length ORFs were subcloned from pENTR™221 entry vectors into the Gateway® destination expression vector, pcDNA3.2/V5-DEST. Bax and nucleophosmin ORFs were subcloned by recombinational cloning (Hartley *et al.*, 2000) which utilises the site-specific recombination properties of bacteriophage lambda to transfer a gene from an Entry clone to a Destination vector to create an Expression clone (Gateway system, Invitrogen). Briefly, 300ng each of Entry and Destination vector plasmid DNA were mixed with LR clonase recombination proteins (Invitrogen) and incubated overnight at 25°C. A positive control reaction for LR recombination was performed using the pENTR-Gus (beta-Glucuronidase) Entry vector. Insertion of Bax and nucleophosmin ORFs into pcDNA3.2/V5-DEST replaces a region previously occupied by the *E.coli* cytotoxic ccdB gene and a Chloramphenicol-resistance (Cm^R) gene. 1µl of each

recombination reaction was used to transform competent DH5α *E. coli* cells and 100µl of transformed cells plated onto LB agar supplemented with 50µg/ml Ampicillin (to positively select transformants containing Amp^R Expression clones) or 30µg/ml Chloramphenicol (to negatively select transformants containing Cm^R Destination vectors). Selected colonies were propagated in competent DH5α *E. coli* cells, plasmid purified and verified by restriction digest using the insert-specific cutting enzyme, Bsu36I (New England Biolabs, Hitchin, Herts, UK).

To enable *in vitro* expression of His-tagged proteins, Bax and nucleophosmin ORFs were subcloned from pENTR™221 into the prokaryotic expression vector, pCR®T7/NT-TOPO, which contains an N-terminal cleavable poly-His tag. Two sets of oligonucleotide primers were designed to amplify the full length Bax and nucleophosmin ORFs to facilitate expression of both tagged and native (untagged) proteins from this vector – see Table 5.1 for primer sequences. Sequences were amplified from pENTR221-Bax and nucleophosmin vectors using a proofreading *Taq* polymerase (Expand High Fidelity Taq; Roche Applied Science) and 30 cycles of PCR (initial melt at 94°C for 2 mins, followed by 10 cycles of 94°C for 15 secs, 45°C for 30 secs and 72°C for 1min, then 20 cycles of the same conditions where the extension step at 72°C was increased by 5 seconds per cycle followed by a final extension at 72°C for 7 mins). The resulting *Taq*-amplified PCR fragments were separated by 1% agarose gel electrophoresis and visualised by ethidium bromide staining under UV illumination. *Taq*-amplified PCR fragments were TOPO-TA cloned into the prokaryotic expression vector pCR®T7/NT-TOPO according to the manufacturer's instructions. Briefly 2µl of the *Taq*-amplified PCR fragments were combined with 1µl salt solution (200 mM NaCl, 10 mM $MgCl_2$), 1µl of linearised pCR®T7/NT-TOPO vector containing covalently-bound topisomerase I and double-distilled water up to a final volume of 6µl. The reaction was then incubated at room temperature for 5mins. pCR®T7/NT-TOPO-Bax and -nucleophosmin clones were transformed into competent DH5α library efficiency *E. coli* cells (Invitrogen) and plated onto LB agar supplemented with 50µg/ml Ampicillin.

Primer	Sequence (5'-3')
1. Bax forward primer for tagged protein	*ATG*GACGGGTCCGGGGAGCA
2. Bax forward primer for native protein	**TAG**AGGAGGAGCAGGCACC*ATG*GA C
3. Bax reverse primer	CTAGCCCATCTTCTTCCAGATGG
4. NPM forward primer for tagged protein	*ATG*GAAGATTCGATGGAGATGG
5. NPM forward primer for native protein	**TAG**AGGAGGAGCAGGCACC*ATG*GAA
6. NPM reverse primer	GCTGGGTTCTAAAGAGACTTCC
7. T7 promotor	GTAATACGACTCACTATAGGGC
8. T7 reverse	CTAGTTATTGCTCAGCGG

Table 5.1. Primer sequences used to TA-clone His-tagged and untagged Bax and nucleophosmin ORFs from pENTR221 to pCRT7/NT-TOPO. Two forward primers to each sequence were used for the sub-cloning of Bax and nucleophosmin in conjunction with a reverse primer. Forward primers for the expression of tagged protein (primers 1 and 4) were designed to start at the ATG initiation codon for the respective sequences and would allow the genes to be cloned into the expression vector in frame with the poly-histidine tag. Forward primers for expression of native protein (primers 2 and 5) were modified by the insertion of a STOP codon (TAG, highlighted in bold blue), followed by the insertion of a ribosome binding site (RBS, AGGAGG, underlined) and the ATG start codon (highlighted in italicised red) of the respective gene to permit translation to re-initiate at the beginning of the cloned gene sequence. Reverse primers were complementary to the 5' end of the ORFs and contained the stop codons (highlighted in green) for each of the genes.

The orientation of the cloned PCR fragments in pCR®T7/NT-TOPO was determined by direct lysis PCR. Briefly, transformed cells were disrupted by heating at 95°C for 5 mins in 20mM Tris pH8.4, 2mM EDTA, 1% Triton X100. After centrifugation at 13000rpm for 2 mins, the supernatant was subjected to the PCR protocol outlined above using either the T7 promoter or T7 reverse primer in conjunction with a gene specific reverse primer to amplify correctly orientated inserts. Plasmid DNA was then purified and correct insert size confirmed by restriction enzyme digestion. pCR®T7/NT-Bax clones were digested with Bsu361, NheI and Hind III, and pCR®T7/NT-nucleophosmin clones with Bsu361, BamHI and HindIII using standard procedures. Clones were also sequenced to confirm error-free recombination (DNA Sequencing Facility, Department of Biochemistry, University of Oxford, UK).

5.3.3 Expression of recombinant full-length Bax and Nucleophosmin proteins

In vitro expression of Eukaryotic Bax and nucleophosmin proteins were performed from the T7 promoter of pcDNA3.2/V5-DEST expression vectors using an *in vitro* quick coupled transcription and translation expression system with rabbit reticulocyte lysate (TnT®; Promega) according to the manufacturer's instructions. Briefly, 1µg purified plasmid DNA was added to a reaction vessel containing a TnT® Quick Master mix (T7 RNA polymerase, nucleotides, salts, amino acids (except methionine), ribonuclease inhibitors and rabbit reticulocyte lysate), to which 1mM methionine and biotin-lysyl-tRNA were added. The TnT® reaction was then incubated at 30°C for 90mins. Inclusion of biotin-lysyl-tRNA incorporated biotinylated lysines into the translated protein for detection using streptavidin-conjugated antibodies. A control reaction using a luciferase-expressing plasmid was also performed. Aliquots (2µl) were subsequently separated by SDS PAGE and visualised by SYPRO ruby staining (Invitrogen) or by Western blotting using a polyclonal Bax antibody (Upstate Biotechnology), a monoclonal nucleophosmin antibody or a streptavidin-HRP conjugate (Promega).

Expression of His-tagged prokaryotic nucleophosmin protein was performed from the T7 promoter of pCR®T7/NT-TOPO using *E.coli* BL21(DE3)pLysS competent cells (Invitrogen). Plasmid DNA was transformed into competent cells and cultures grown overnight at 37°C in Luria-Bertani (LB) broth containing 100μg/ml Ampicillin and 34μg/ml Chloramphenicol. 10ml Fresh LB broths supplemented with antibiotics were inoculated with 500μl of overnight culture and grown to mid-log phase (until culture density had reached an A_{600} of 0.5-0.8). Protein expression was then induced using 1mM Isopropylthiogalactoside (IPTG). A pilot protein expression study was performed, where 0.2ml aliquots of culture (maintained at 37°C) were taken at 2 h intervals up 6 h from the time of induction. Maximal expression of His-tagged nucleophosmin was achieved 4h post induction. His-tagged nucleophosmin was extracted from BL21 cells using a 1 in 10 dilution of Fast–break lysis buffer (Promega). Purification of His-nucleophosmin was performed by incubating samples with MagneHis™-Nickel beads (Promega) for 2 mins to isolate His-tagged protein. Following incubation, beads were captured using a magnetic stand and bound protein washed 4 times in in MagneHis™ binding/wash buffer wash. Protein was eluted in 500mM imidazole and dialysed against Tris Buffer (50mM Tris-HCl pH7.4, 150mM NaCl, 1mM EDTA, 5mM DTT, 0.5% Nonidet P40 (NP40), 0.5% Triton-X-100, 10% glycerol) using Slide-A-Lyzer cassettes (10KDa molecular weight cut-off; Pierce, Northumberland, UK). Aliquots of recombinant human nucleophosmin were separated by SDS PAGE gel electrophoresis and visualised by coomassie staining. Briefly, gels were fixed for 30min in fix solution (40% methanol/7% acetic acid), stained overnight in Brilliant Blue G-colloidal solution (Sigma), de-stained for 1min in de-stain solution (25% methanol/10% acetic acid) and transferred into a 25% methanol solution for imaging. Expressed protein was also analysed by Western blot using a monoclonal nucleophosmin antibody, as described in general materials & methods (Chapter 2), and identified by MALDI-TOF mass spectrometry as described in sections 4.3.5 and 4.3.6. Stock solutions of His-nucleophosmin (2.5mg/ml) were stored at -70°C.

His-tagged prokaryotic Bax was expressed from the T7 promoter of pCR®T7/NT-TOPO using the Expressway™ Plus *in vitro* expression system (Invitrogen) according to the manufacturer's instructions. Briefly, 1µg plasmid DNA was added to a reaction vessel containing 20µl *E.coli* cell extract, 20µl reaction buffer, 1µl T7 enzyme mix and 75mM methionine. Reaction vessels were shaken at 300rpm in an incubator at 37°C for 4h. 5µl RNase was then added and reaction vessels incubated for a further 15mins before undergoing purification. Scaling up the production of His-Bax involved performing 20 replicate reactions. Purification of His-tagged proteins was performed as for His-nucleophosmin, except MagZ Nickel-coated binding beads (Promega) were used. Following dialysis, purified His-Bax was concentrated to approx 2mg/ml using vivaspin concentrators (Vivascience, Hannover, Germany) and biotinylated using the ProtOn™ biotin labelling kit (Vector laboratories, Peterborough, UK) according to the manufacturer's instructions. Aliquots of recombinant human Bax were separated by SDS PAGE gel electrophoresis, visualised by coomassie staining and by Western blot using a polyclonal Bax antibody (Upstate Biotechnology), and identified by MALDI-TOF mass spectrometry. Stock solutions of His-Bax were stable if stored at 4°C for up to one week.

5.3.4 Analysis of interaction between recombinant proteins using an *in vitro* amplified luminescence proximity assay

Interaction studies between recombinant full-length human Bax and nucleophosmin and were performed using a bead-based chemiluminescent proximity assay (AlphaScreen™ technology; Perkin Elmer) on 384-well white polystyrene proxiplates (Perkin Elmer). This work was carried out in collaboration with Dr Jane Thompson and Mrs Eliane Salvo-Chirmside (ACE).

Acceptor beads were conjugated with a monoclonal antibody raised against the C-terminus of nucleophosmin (Sigma) using a standard amination procedure. 0.3mg acceptor beads were combined with 30µg antibody in a reaction mix containing 5%

sodium cyanoborohydride and 0.02M 2-[N-Morpholino] ethanesulphonic acid (MES; pH6.0) and incubated for 48h at 37°C in the dark. Following incubation, a block step was performed using 0.1M carboxymethoxylaminehemihydrochloride (CMO) for 1h. The beads were then sonicated and washed 3 times in 0.1M Tris buffer, pH8.0. Conjugated beads were finally prepared as a 5mg/ml solution in 25mM HEPES, 0.1M NaCl, pH 7.4 (Perkin Elmer). Confirmation of successful conjugation was assessed by combining conjugated acceptor beads with streptavidin-conjugated donor beads (PerkinElmer) in reactions containing serial dilutions (0.003-3nM) of biotinylated mouse secondary antibody (Sigma).

Assay reactions comprised 5µl each of nucleophosmin monoclonal antibody-conjugated acceptor beads and streptavidin-coated donor beads, 15µl recombinant biotinylated Bax and nucleophosmin proteins diluted to 100nM and 15nM, respectively, in 150mM NaCl, 10mM HEPES-KOH pH7.4, 0.01% BSA containing 0.1% NP40, with inhibitors as indicated, in a total assay volume of 25µl. Prior to contact with beads, recombinant proteins were mixed together for 90mins at room temperature to encourage interaction. For experiments containing inhibitors to either Bax or nucleophosmin, protein-inhibitor solutions were pre-incubated separately at room temperature for 30mins prior to mixing and incubating a further 90mins. Optimisation of reaction conditions included protein concentrations used (0-5000nM titration of Bax protein to which was added either 5nM, 15nM, 150nM or 350nM nucleophosmin protein) and pre-assay incubation times (a range of 1min to 240min was used).

The proximity assay was performed as follows: In a darkened room, nucleophosmin antibody-conjugated acceptor beads were added to 384-well plates followed by recombinant proteins and plates incubated in the dark at 37°C for 30mins. Streptavidin-conjugated donor beads were then added and plates incubated for a further 60mins prior to analysis using a multi-label reader (Envision Excite™; Perkin Elmer).

Peptide inhibitors used were Bcl-2 (human, His-tagged, C-terminal truncated; Merck, Nottingham, UK) and p53 peptide (residues 277-296; Abcam). Antibody-based inhibitors used were raised against the Bax N-terminus (residues 1-21; Upstate Biotechnology), α-helix 5 (residues 98-117, Ab4; Merk) and C-terminus (residues 150-165; Abcam). Inhibitor concentrations were titrated from 0.03nM to 1000nM when included in the proximity assay.

Control reactions containing nucleophosmin antibody-conjugated acceptor beads and streptavidin-conjugated donor beads with nucleophosmin or Bax alone, were run in all experiments to assess background signal intensity.

5.4. Results

5.4.1. Production of full-length recombinant human Bax and nucleophosmin proteins

Confirmation that the pENTR221 Ultimate human ORF clones supplied by Invitrogen contained the full-length Bax and nucleophosmin ORFs was assessed by restriction digest – see Figure 5.3. In order to express sufficient quantities of recombinant Bax and nucleophosmin for use in proximity assay interaction studies, human Bax and nucleophosmin full-length ORFs were subcloned from pENTR221 into dedicated expression plasmid DNA vectors – see Figure 5.4 for a flow-diagram of the subcloning strategies used.

Eukaryotic expression constructs for Bax and nucleophosmin were produced by subcloning full-length ORFs from pENTR221 into the mammalian expression vector pcDNA3.2/V5-DEST by recombinational cloning. In order to confirm that these expression clones contained full length ORFs, recombinant Bax and nucleophosmin were expressed from the T7 promoter of pcDNA3.2/V5-DEST using a cell-free expression system (TnT®) – see Figure 5.5. In the TnT® reaction, plasmid DNA was combined with T7 RNA polymerase and rabbit reticulocyte cell lysate to initiate eukaryotic mRNA translation (Jackson and Hunt, 1983; Craig *et al.*, 1992). Nascent proteins were labelled by including biotinylated lysine in the TnT® reaction mix and visualised following SDS PAGE by Western blotting with a streptavidin-HRP-conjugated secondary antibody. A clear signal of anticipated molecular weight was obtained for the nucleophosmin-expressing clones, however no signal could be detected for Bax – see Figure 5.5a. The inability to detect biotinylated recombinant Bax by streptavidin-HRP could be explained by the respective number of lysine residues contained in each protein. Lysine is one of the more frequently used amino acids and on average represents 6.6% of the amino acid content of a protein (Dayhoff, 1978). In this

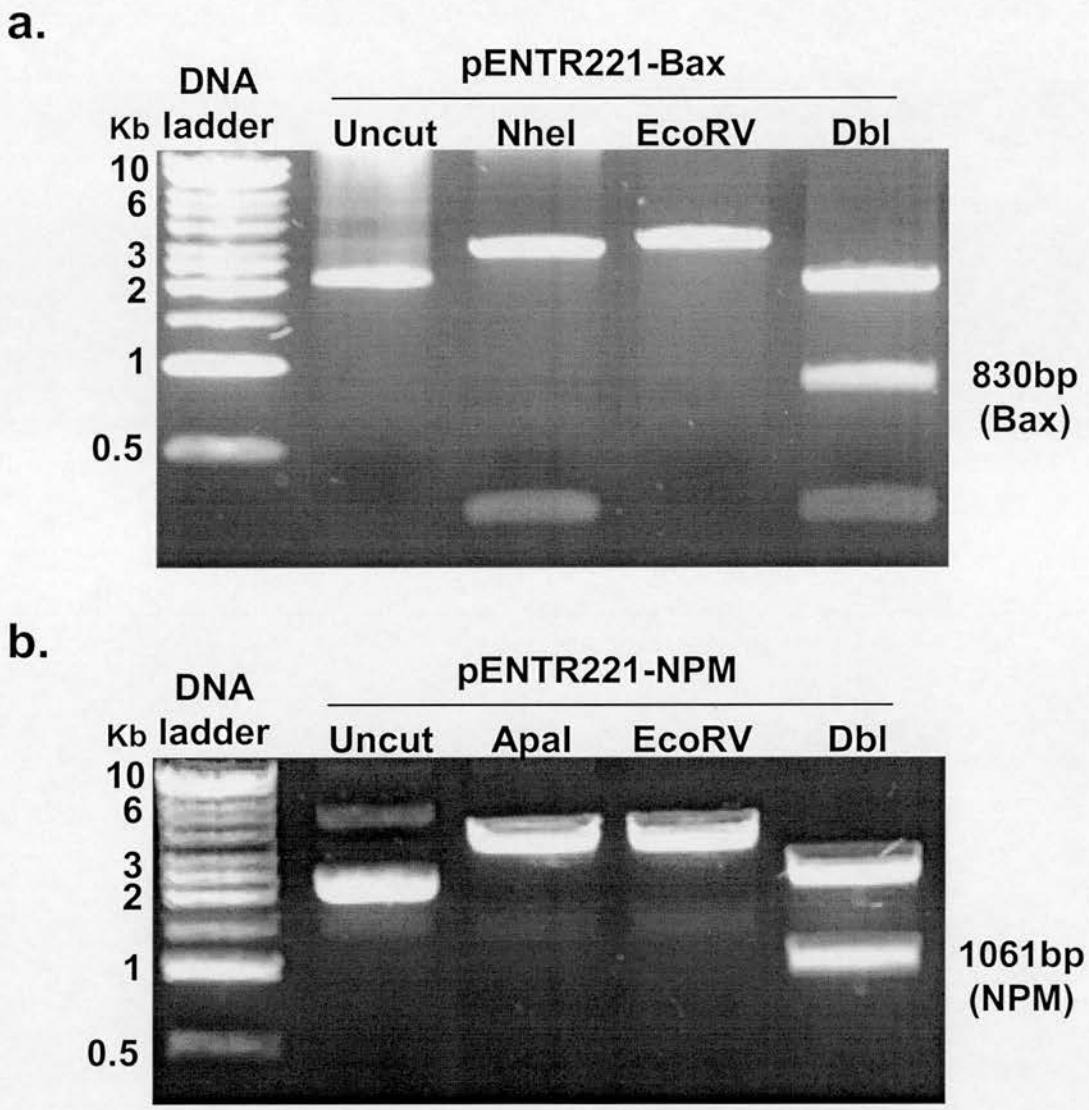


Figure 5.3. Validation of pENTR221 full-length Bax and nucleophosmin ORF clones by restriction digest. Single digests with the restriction enzymes EcoRV andNheI (pENTR221-Bax) **(a)** and ApaI (pENTR221-nucleophosmin(NPM)) **(b)** linearised the plasmid DNA clones, whereas a double digest (DbI) generated a 830bp fragment (pENTR221-Bax) and a 1061bp fragment (pENTR221-NPM) containing the full length Bax and nucleophosmin ORFs, respectively. Uncut contains undigested plasmid DNA.

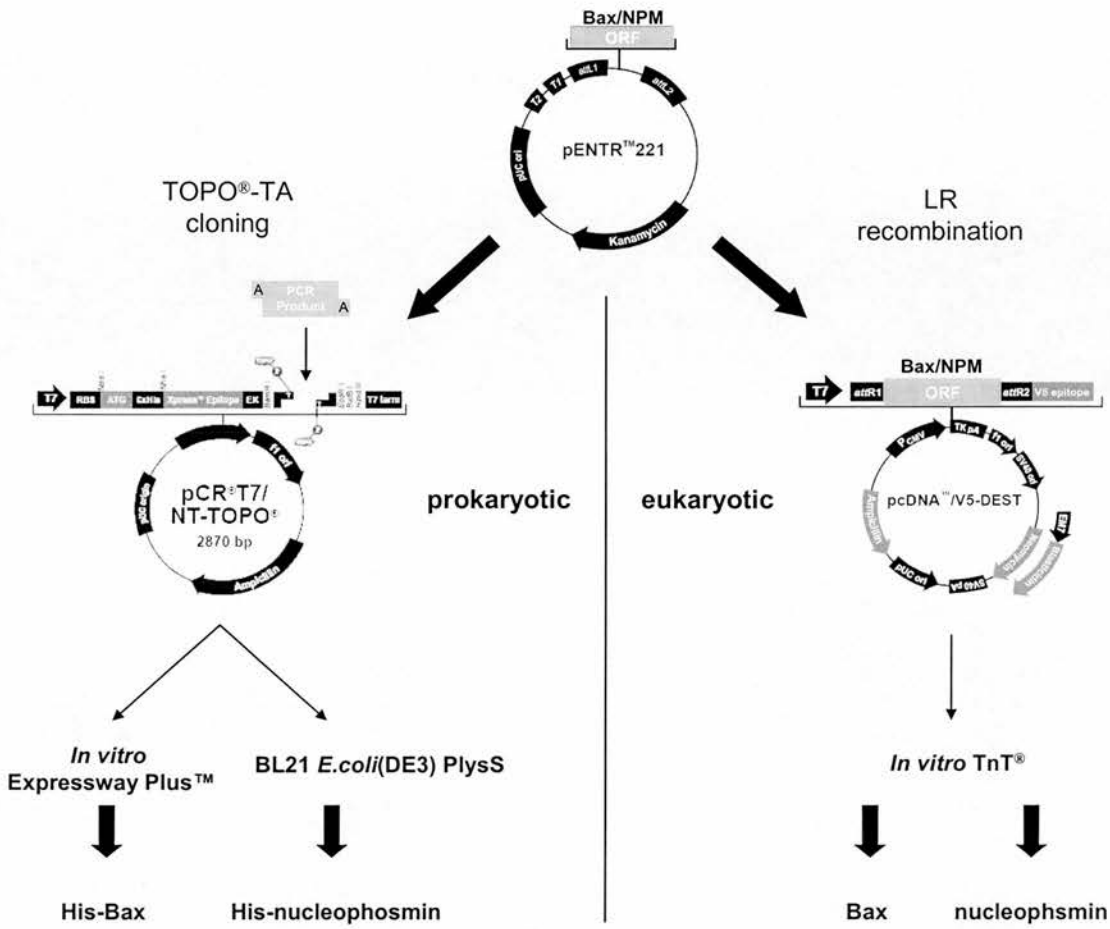


Figure 5.4. Sub-cloning strategy to produce recombinant Bax and nucleophosmin proteins for use in proximity assay. Full length Bax and nucleophosmin open reading frames (ORFs) were supplied in the pENTR221 vector. Bax and nucleophosmin ORFs were sub-cloned into pcDNA3.2/V5-DEST by LR recombination or into pCRT7/NT-TOPO® by TOPO®-TA cloning. Eukaryotic proteins were expressed from the CMV promotor of pcDNA3.2/V5-DEST using a cell-free *in vitro* transcription and translation (TnT®) system. Prokaryotic His-tagged proteins were expressed from the T7 promoter of pCRT7/NT-TOPO® using the Expressway Plus™ cell-free *in vitro* expression system (Bax) or in IPTG-inducible BL21 *E. coli* cells (nucleophosmin). Proteins were then purified before interaction between them was assessed by proximity assay.

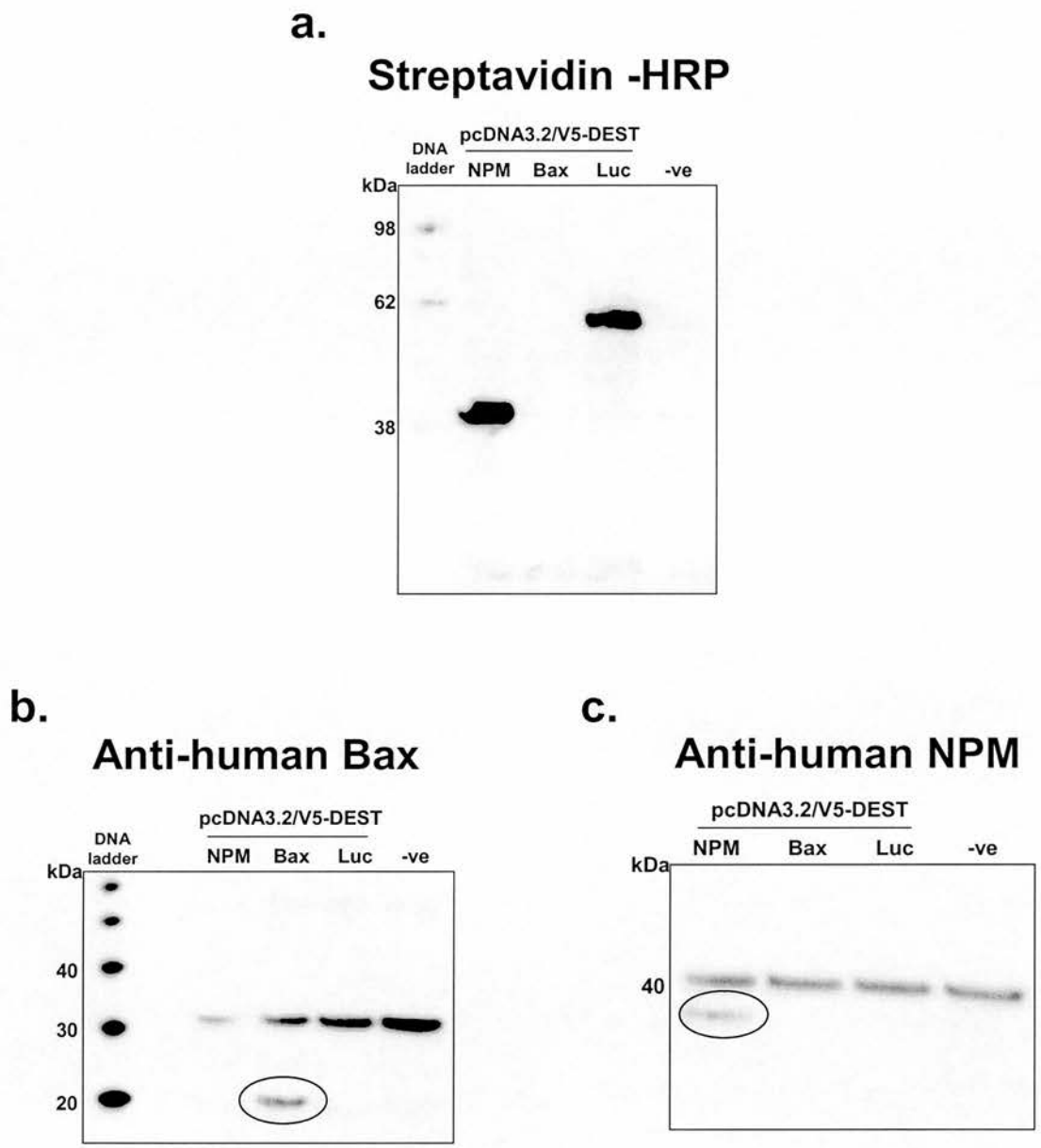


Figure 5.5. Analysis of Bax and nucleophosmin protein expression from pcDNA3.2/V5-DEST by Western blot. TnT® - expressed proteins were separated by SDS PAGE and analysed for the presence of Bax and nucleophosmin (NPM) by Western blot. Detection of biotinylated proteins was performed using a streptavidin-HRP antibody **(a)**. Western blots were also probed with antibodies raised against human Bax **(b)** and nucleophosmin **(c)**. Although only biotinylated nucleophosmin was visible on the streptavidin blot, both proteins could be detected by their respective human-reactive antibodies (circled). Non-specific protein bands at 32kDa and 40kDa in the Bax and nucleophosmin blots, respectively, originate from constituents within the TnT® reaction. Positive control reactions expressing luciferase (Luc) and negative control reactions (-ve) containing an empty vector were also run.

respect, nucleophosmin is above average, with 33 lysines out of a total amino acid content of 294 (11.2%). In contrast, Bax contains only 9 lysines out of 192 amino acids (4.7%) which may limit its detection using biotinylated lysines. However, both Bax and nucleophosmin were detected at appropriate molecular weights with human protein-specific antibodies by Western blot, demonstrating that full-length eukaryotic Bax and nucleophosmin proteins had indeed been expressed – see Figures 5.5b & c.

In order to determine the yield of proteins expressed by the *in vitro* TnT® reactions, proteins were separated by SDS PAGE and protein band intensity visualised by SYPRO ruby stain – see Figure 5.6. Although expression of recombinant proteins was detectable by Western blot, protein levels were too low to be visualised by this stain. The minimal detection threshold for SYPRO ruby stain is approximately 1ng (Berggren *et al.*, 2000), suggesting that the protein yield generated by *in vitro* expression of eukaryotic Bax and nucleophosmin by a single TnT® reaction was less than this. Therefore, to increase protein yield to a level sufficient for experimental use (ideally in the order of milligrams), an alternative subcloning protocol was undertaken.

To maximise yield and purity of recombinant proteins, His-tag fusion proteins were produced in *E.coli* cultures. To facilitate this, PCR primers were designed to isolate and amplify Bax and nucleophosmin ORFs from pENTR221 using a high fidelity *Taq* polymerase and transfer them by means of TOPO-TA cloning into the dedicated prokaryotic expression vector, pCR®T7/NT-TOPO. The forward primers were designed in such a way as to either permit addition of an N- terminal 6xHis-tag (by placing the gene of interest in frame with the His-tag) or to express native proteins (by including a STOP codon and a new ribosome binding site (RBS) to facilitate expression from the initial ATG of the inserted gene). *Taq* polymerase has a terminal transferase activity that adds a single 3' deoxyadenosine (A) overhang to each end of the generated PCR fragments that facilitates their ligation into linearised pCR®T7/NT-TOPO plasmid DNA containing single 3' deoxythymidine (T) overhangs. Although fast and effective, TA-cloning is non-directional. Therefore, correct orientation of the inserted PCR fragment

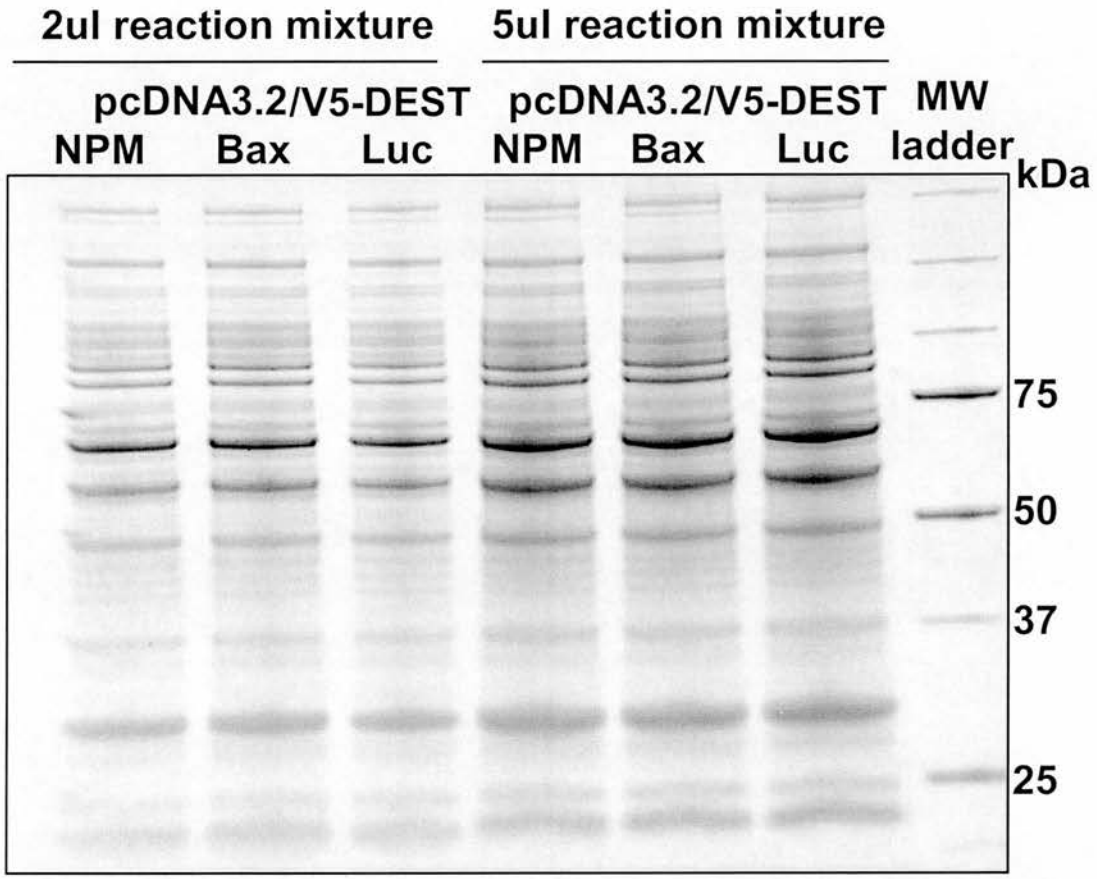


Figure 5.6. Visualisation of recombinant Bax and nucleophosmin protein expression from pcDNA3.2/V5-DEST by SYPRO ruby stain. 2µl and 5µl of each TnT® reaction were separated by SDS PAGE and protein content visualised by SYPRO ruby stain. Despite confirmation of Bax and nucleophosmin protein expression by Western blot (Figure 5.5), expression levels were too low to be evident by protein stain. Visible bands are components of the rabbit reticulate lysate used in the TnT® reaction.

into pCR®T7/NT-TOPO was confirmed by PCR using T7 forward (T7F) and reverse (T7R) primers in combination with specific Bax or nucleophosmin reverse primers. The rationale behind this was that if the inserted DNA fragment was successfully amplified from pCR®T7/NT-TOPO using the T7F primer, then the insert was correctly orientated. However, if the DNA fragment was amplified using the T7R primer, then the insert was in the reverse orientation – see Figures 5.7. In order to confirm that these clones contained correctly aligned full-length His-tagged ORFs, recombinant His-tagged Bax and nucleophosmin were expressed using a prokaryotic *in vitro* expression system, expressed proteins separated by SDS PAGE and detected with specific antibodies by Western blot – see Figure 5.8.

Scaling-up the expression of His-tagged Bax and nucleophosmin was then attempted using IPTG-inducible BL21 *E.coli* cultures. Although high yields (in the order of milligrams) of recombinant nucleophosmin were produced, expression of Bax in this system was unsuccessful as Bax over-expression was toxic to *E.coli*. Milligram amounts of recombinant Bax were subsequently obtained by performing repeated *in vitro* prokaryotic expression reactions. His-tagged proteins were purified using Nickel-chelated purification columns, separated by SDS PAGE and visualised by coomassie stain – see Figure 5.9. Recombinant proteins were confirmed as human Bax and nucleophosmin by MALDI-TOF mass spectrometry – see Figure 5.10.

5.4.2. Establishing the cell-free amplified luminescent proximity assay

A cell-free amplified luminescent proximity assay was developed to investigate the interaction between recombinant Bax and nucleophosmin proteins at the molecular level. Both halves of the proximity assay were established by attaching biotinylated Bax protein to streptavidin-coated donor beads and monoclonal nucleophosmin antibody to acceptor beads – see Figure 5.11a. The presence of biotinylated Bax on the donor beads was confirmed by the inability of these beads to interact with biotin-coated acceptor beads – see Figure 5.11b. To confirm the conjugation of acceptor beads with the

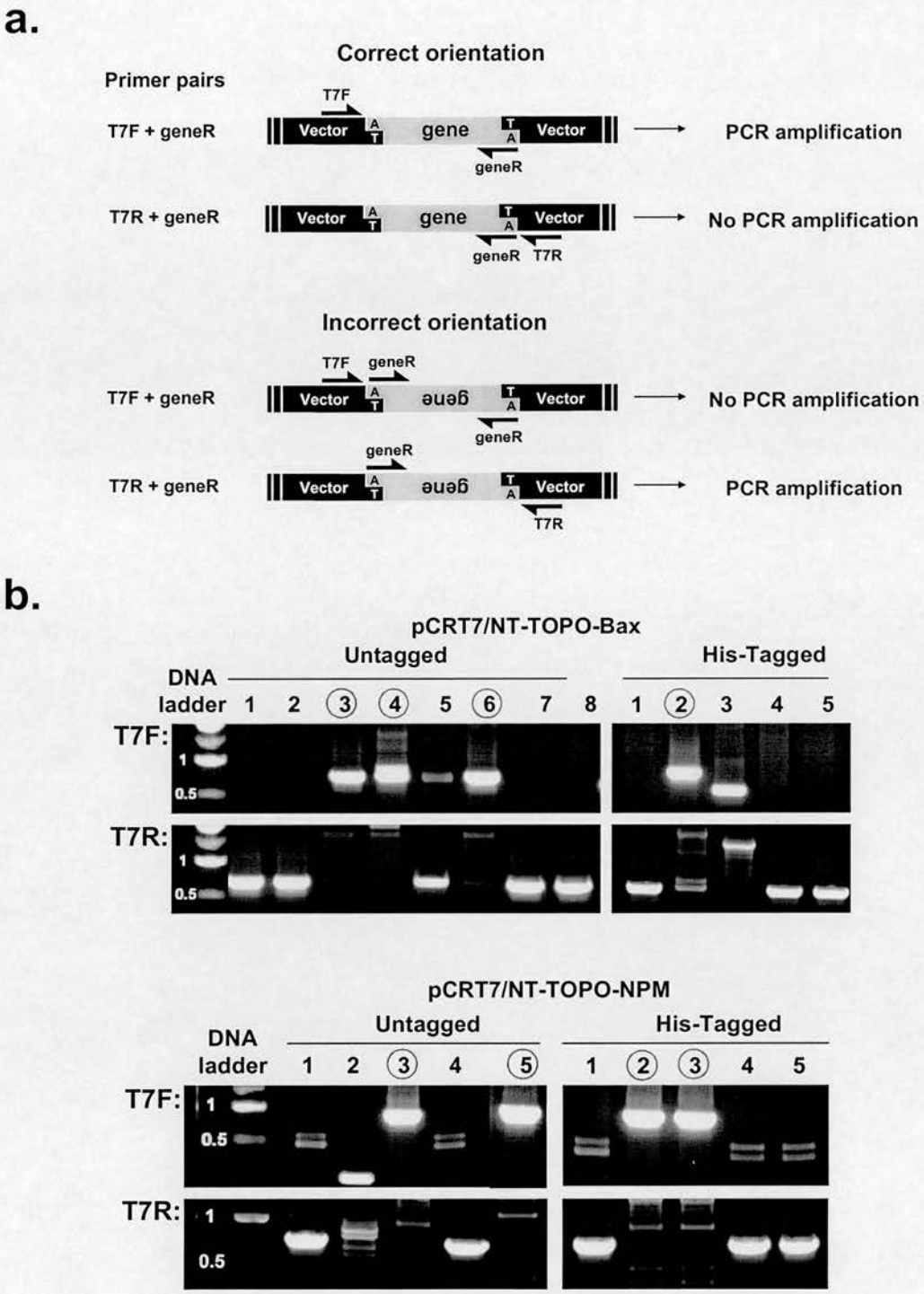


Figure 5.7. Analysis of Bax and nucleophosmin insert orientation in pCRT7/NT-TOPO by direct lysis PCR. In the schematic model shown in **(a)**, correctly orientated gene inserts are amplified by PCR using a T7 forward (T7F) and gene-specific reverse (geneR) primer pair, but not when a T7 reverse (T7R) primer is used. Where the gene is inserted incorrectly, the reverse is true. Direct lysis PCR of several pCRT7/NT-TOPO-Bax **(b)** and –nucleophosmin **(c)** colonies using either T7F/geneR or T7R/geneR primer pairs identified correctly orientated gene inserts, as indicated by those colony numbers circled in red.

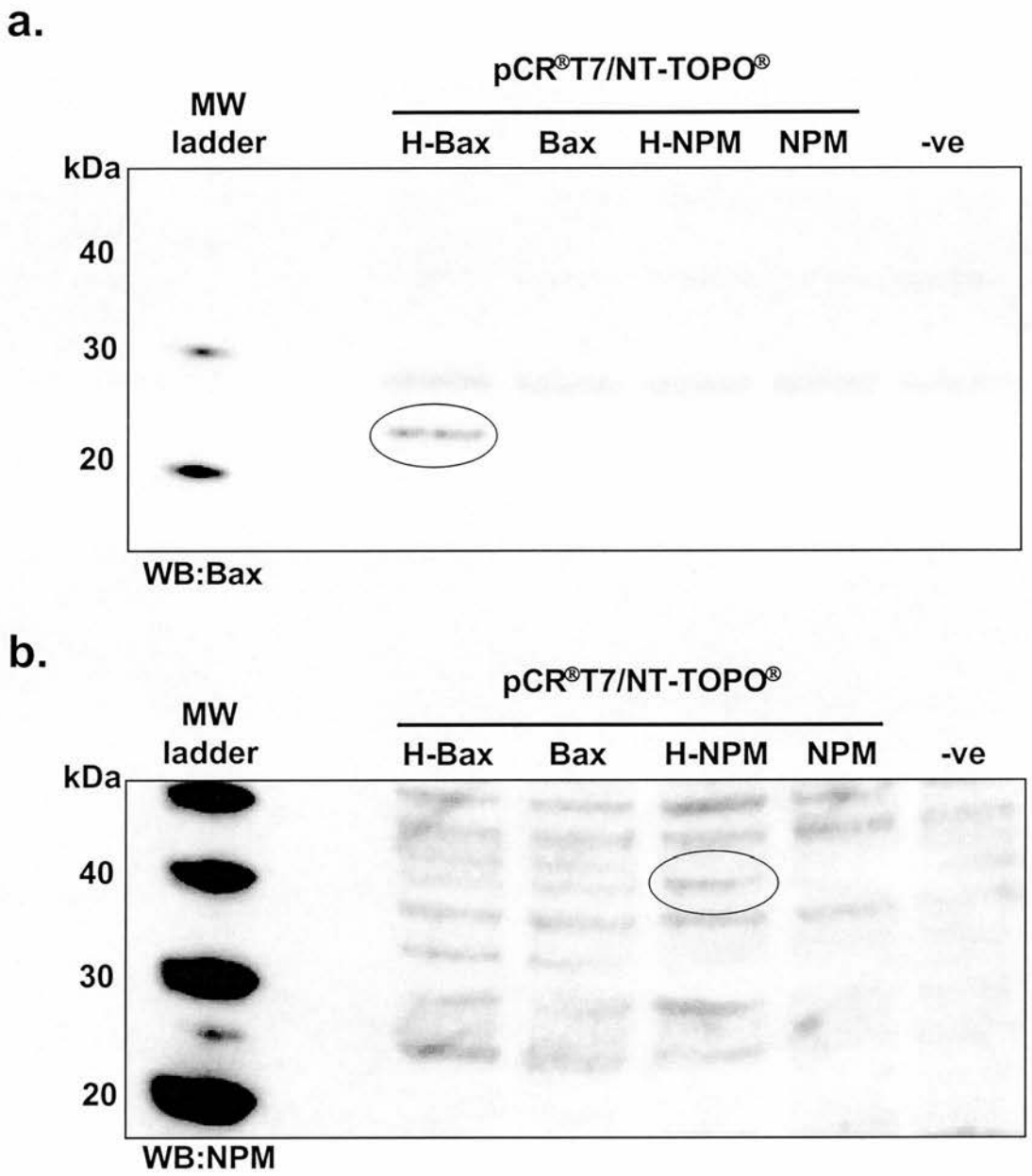


Figure 5.8. Confirmation of *in vitro* expression of His-tagged Bax and nucleophosmin from pCR®T7/NT-TOPO by Western blot. Native and His-tagged Bax and nucleophosmin proteins were expressed from pCR®T7/NT-TOPO using an *in vitro* prokaryotic expression system. Reaction samples were separated by SDS-PAGE and visualised by Western blot for the presence of Bax **(a)** and nucleophosmin **(b)** using antibodies raised against respective human proteins. His-Bax (H-Bax; 26kDa) and His-nucleophosmin (H-NPM; 42kDa) are circled on the respective blots. Empty-vector reactions were also run as negative controls (-ve).

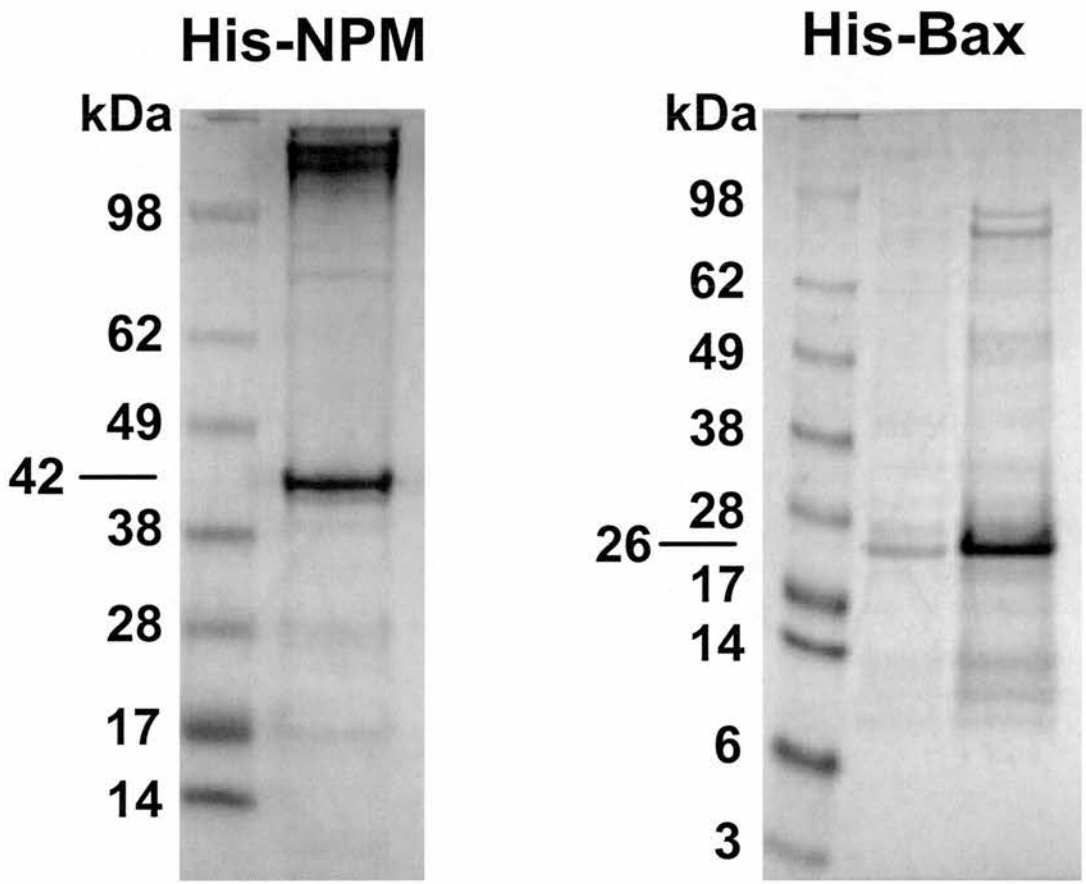
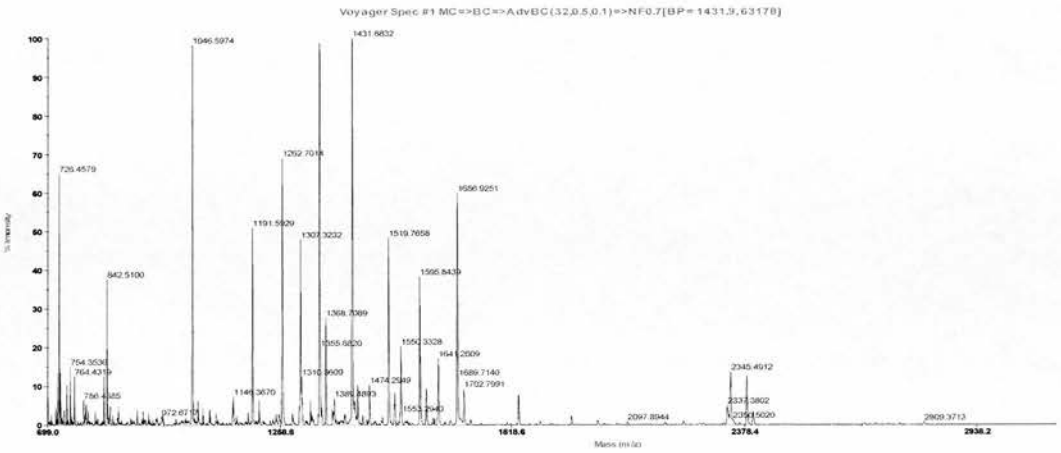


Figure 5.9. Expression of purified His-tagged prokaryotic nucleophosmin and Bax. His-tagged nucleophosmin (42kDa) was expressed in BL21 pLysS cells from pCRT7/NT-TOPO; His-tagged Bax (26kDa) was *in vitro* expressed using the Expressway plus system. Purified proteins were analysed by SDS-PAGE gel electrophoresis and coomassie stained.

a.

His-Bax



b.

His-nucleophosmin

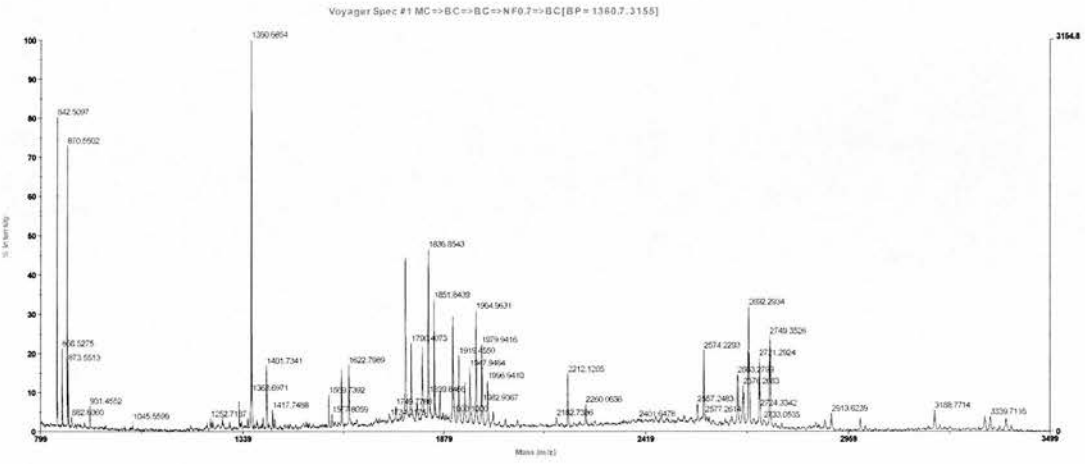


Figure 5.10. Identification of recombinant proteins by MALDI-TOF mass spectrometry. Purified His-tagged recombinant proteins were in-gel trypsin digested and analysed by MALDI-TOF mass spectrometry. Peptide masses from the above mass spectra confirmed that the expressed proteins were human Bax **(a)** and human nucleophosmin **(b)**.

nucleophosmin antibody, a biotinylated secondary antibody was used to link the acceptor beads to streptavidin-coated donor beads, thus generating a signal – see Figure 5.11c.

Having verified both halves of the proximity assay, experiments were performed to determine the optimal assay conditions. Optimisation of interacting protein concentrations was achieved by performing dose response experiments. Bax (0.05nM – 5000nM) produced a concentration-dependent increase in interaction signal when incubated with constant concentrations of nucleophosmin (5nM, 15nM, 150nM and 350nM) - see Figure 5.12a. Interaction signal reached a maximum level when 5nM and 15nM nucleophosmin was added. At higher concentrations of nucleophosmin (150nM and 350nM) maximal signal intensity decreased by approximately 30% and 40%, respectively, compared to the highest signal observed, probably due to saturation of antibody binding sites on the acceptor beads. Combining 15nM nucleophosmin with increasing concentrations of Bax identified a maximal interaction signal when 100nM Bax was used – see Figure 5.12b. Time-course studies identified a time-dependent increase in interaction signal that reached a maximal plateau at 90min – see Figure 5.12c. Thus, in all subsequent experiments, 15nM nucleophosmin and 100nM Bax were incubated together for at least 90mins prior to beginning the proximity assay.

5.4.3. Nucleophosmin preferentially interacts with detergent-activated Bax

In order to determine whether the Bax-nucleophosmin interaction depends on the activation state of Bax, the proximity assay was used to study the interaction between Bax and nucleophosmin in the presence of the conformational change-inducing detergent NP-40 or the non-modifying detergent CHAPS – see Figure 5.13. When nucleophosmin was combined with Bax that had been pre-incubated with CHAPS, no appreciable interaction signal was generated. However, when nucleophosmin was mixed with Bax that had been pre-incubated with NP-40, a strong interaction signal was observed – see Figure 5.13a. A widely used indicator of this apoptosis/detergent-

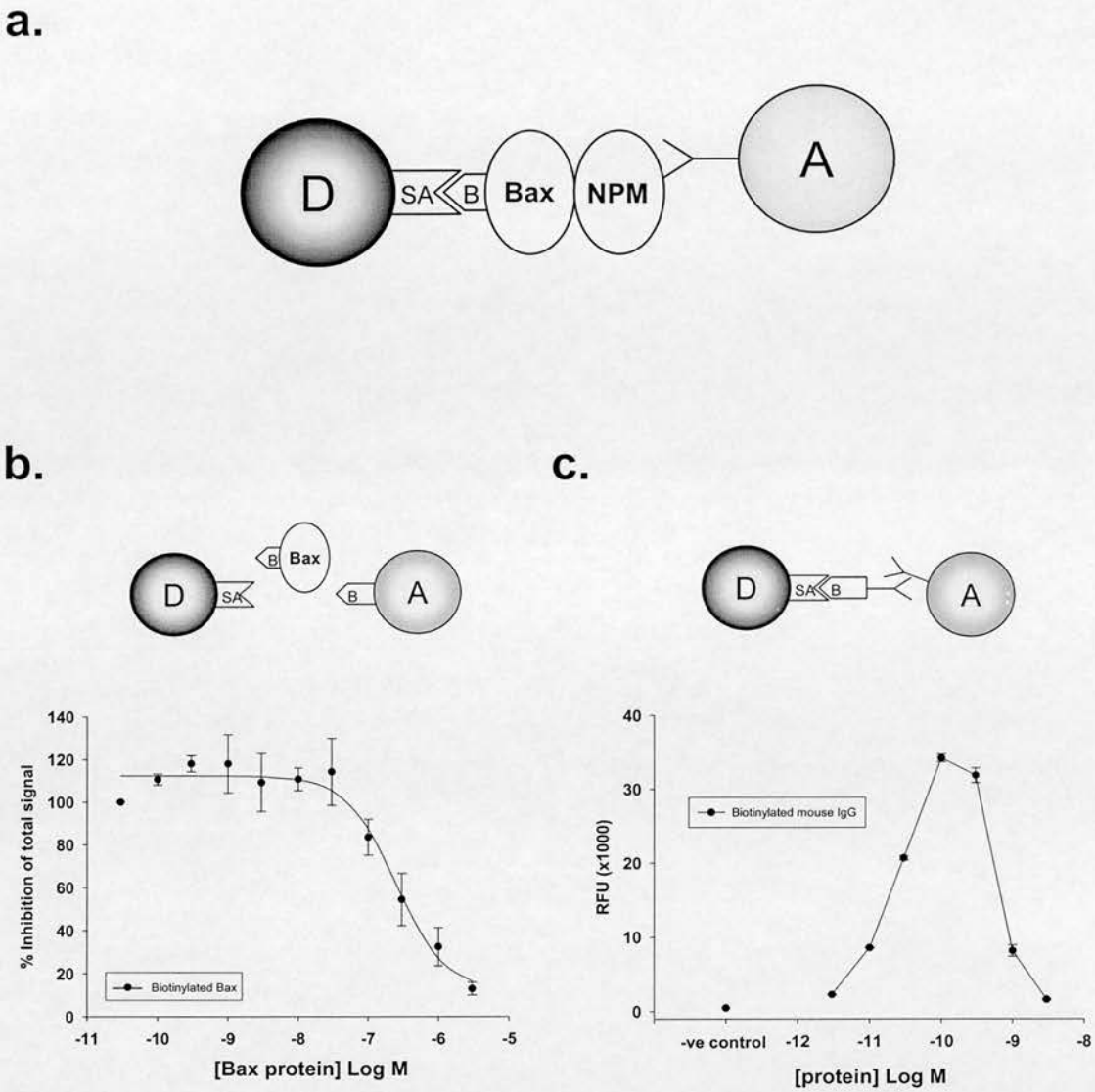


Figure 5.11. Establishing a cell-free proximity assay to investigate the interaction between recombinant Bax and nucleophosmin. An *in vitro* amplified luminescent proximity assay consisting of streptavidin (SA)-conjugated donor beads (D), monoclonal nucleophosmin antibody-conjugated acceptor beads (A) and biotinylated (B) recombinant Bax (Bax) and nucleophosmin (NPM) proteins was established as depicted in **(a)**. Binding of biotinylated Bax to SA-donor beads was confirmed by performing a competition assay with biotinylated acceptor beads **(b)**. Conjugation of acceptor beads with monoclonal nucleophosmin antibodies was confirmed using a biotinylated secondary antibody to link conjugated acceptor beads with SA-donor beads to generate an interaction signal **(c)**. Data represent mean ± s.e.m of three independent experiments.

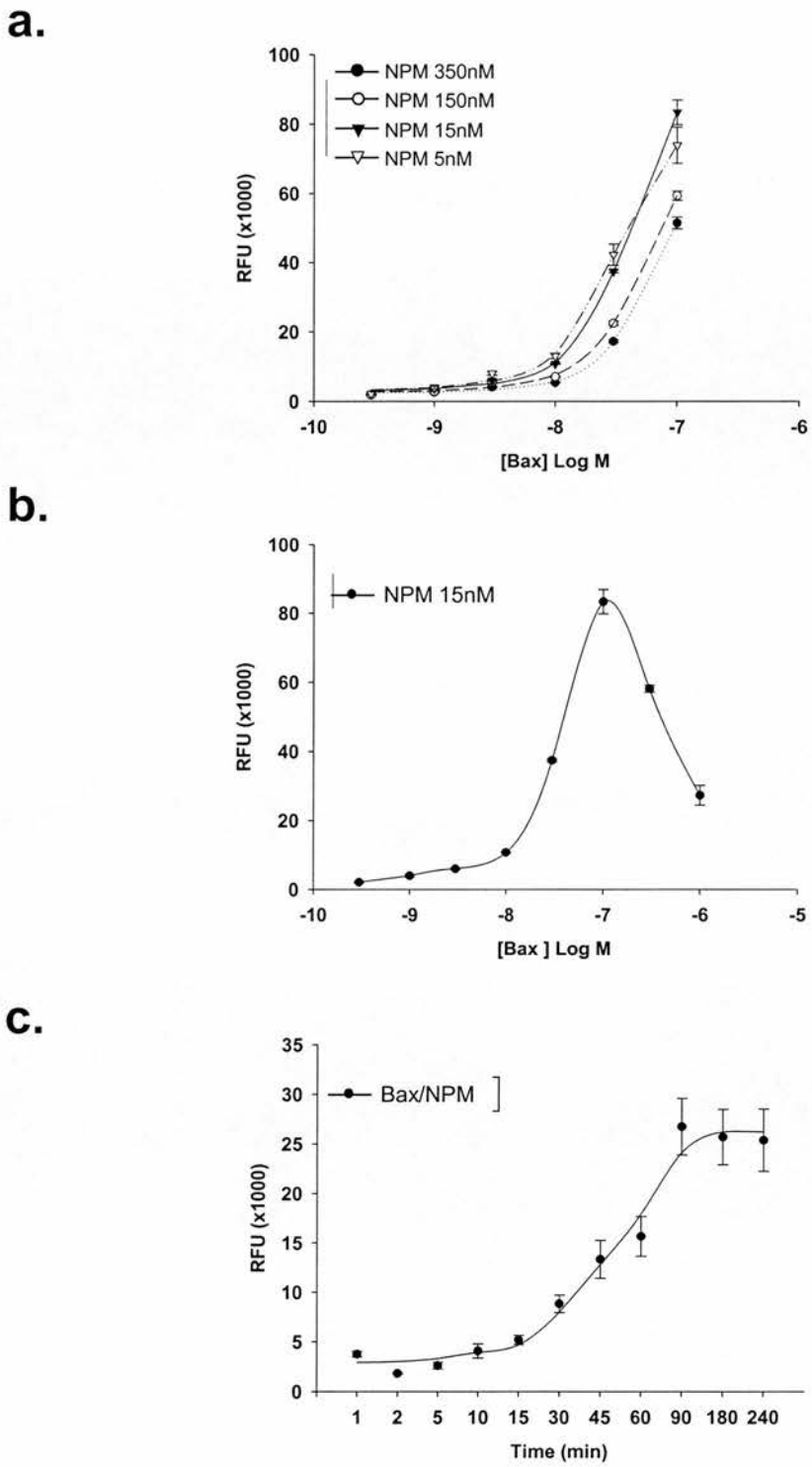
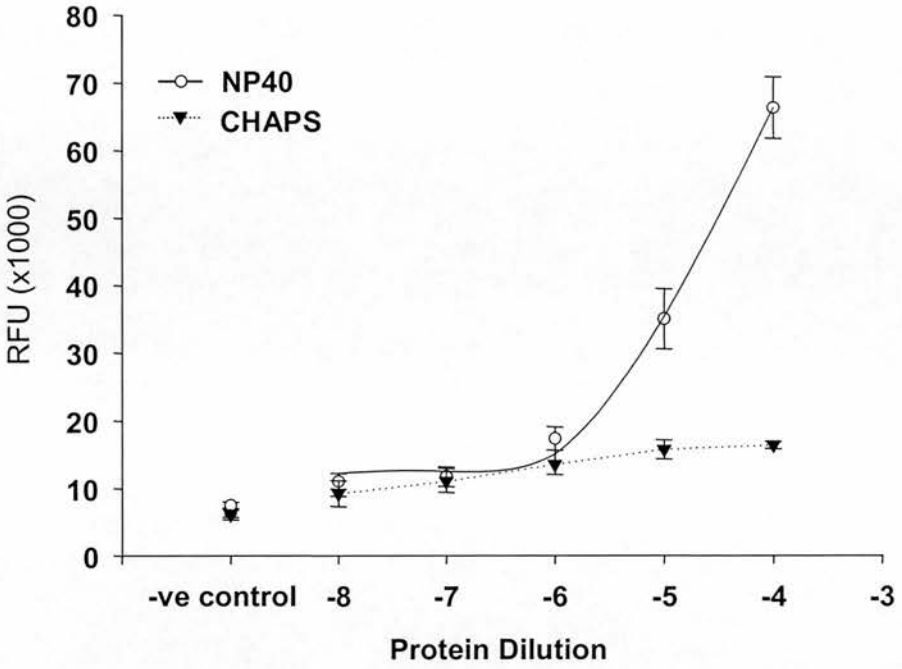


Figure 5.12. Optimising proximity assay reaction conditions. Concentration response experiments using recombinant Bax and nucleophosmin proteins were performed to identify optimal stoichiometry for maximal interaction signal generation **(a)**. Reactions containing 15nM nucleophosmin gave the highest signal which peaked when combined with 100nMBax **(b)**. Time-course experiments identified an incubation time of 90mins as optimal **(c)**. Data represent mean ± s.e.m of three independent experiments.

a.



b.

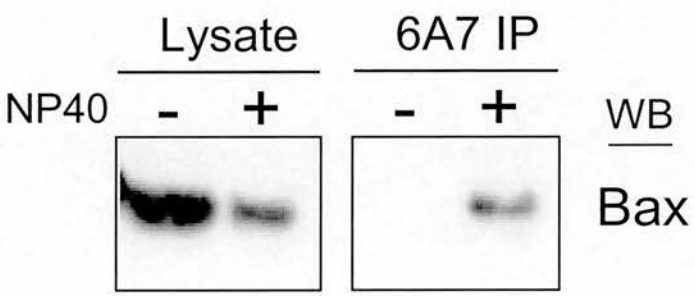


Figure 5.13. Interaction between recombinant Bax and nucleophosmin proteins in the presence of detergents. Serial dilutions of Bax and nucleophosmin were mixed in the presence of either 0.1% CHAPS or NP-40 and interaction measured by proximity assay. Maximal interaction between the recombinant proteins was obtained with the Bax conformational change-inducing detergent, NP-40, whereas in the presence of the non-modifying detergent, CHAPS, little interaction was seen **(a)**. Bax conformational change was assessed by immunoprecipitating Bax from the NP40 detergent-containing reaction using the conformation sensitive Bax 6A7 antibody **(b)**. Data represent mean $\pm$ s.e.m of three independent experiments.

induced structural change in Bax is to demonstrate immunoreactivity with a Bax antibody recognising the normally concealed N-terminal 6A7 epitope (Hsu and Youle, 1997). That exposure to NP-40 had induced this conformational change in Bax was confirmed by immunoprecipitating 6A7 reactive Bax exclusively from the detergent-treated reaction – see Figure 5.13b. Thus, NP-40 was included in the assay buffer in all subsequent experiments.

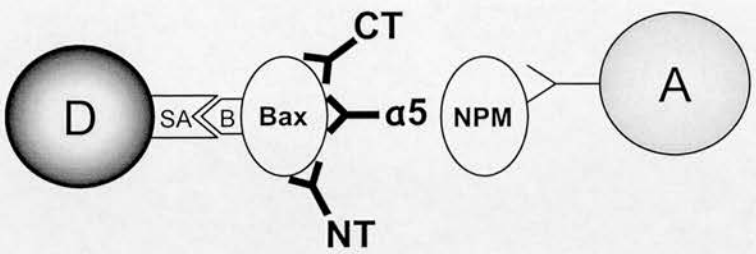
5.4.4. Nucleophosmin interacts with the C-terminus of Bax but not with the N-terminus or pore-forming domain

To determine whether nucleophosmin binds to additional regions within the Bax protein structure other than the C-terminal domain, recombinant Bax was pre-incubated with several Bax-specific antibodies recognising distinct regulatory domains before being incubated with nucleophosmin and interaction measured by proximity assay. Antibodies recognising epitopes within the Bax N-terminus (residues 1-21), α-helix 5 of the pore-forming domain (Ab4; residues 98-117) and C-terminus (residues 150-165) were used as inhibitors. Addition of the Bax C-terminal-binding antibody inhibited the interaction between Bax and nucleophosmin with an apparent IC_{50} of 1.43±0.65nM whereas neither the N-terminal nor the pore-forming-binding antibodies were able to inhibit the interaction signal – see Figure 5.14.

5.4.5. Interaction between Bax and nucleophosmin is inhibited by p53 and Bcl-2 peptides

Putative inhibitors to Bax and nucleophosmin were added to the reaction mixtures in order to gain further insight into the domains forming the interface between Bax and nucleophosmin. Peptides derived from Bcl-2 (which interacts with Bax via its BH3 domain (Zha *et al.*, 1996) and p53 (known to interact with the C-terminal domain of nucleophosmin (Colombo *et al.*, 2002; Lambert and Buckle, 2006)) were pre-incubated separately with recombinant Bax and nucleophosmin proteins, and their impact on the

a.



b.

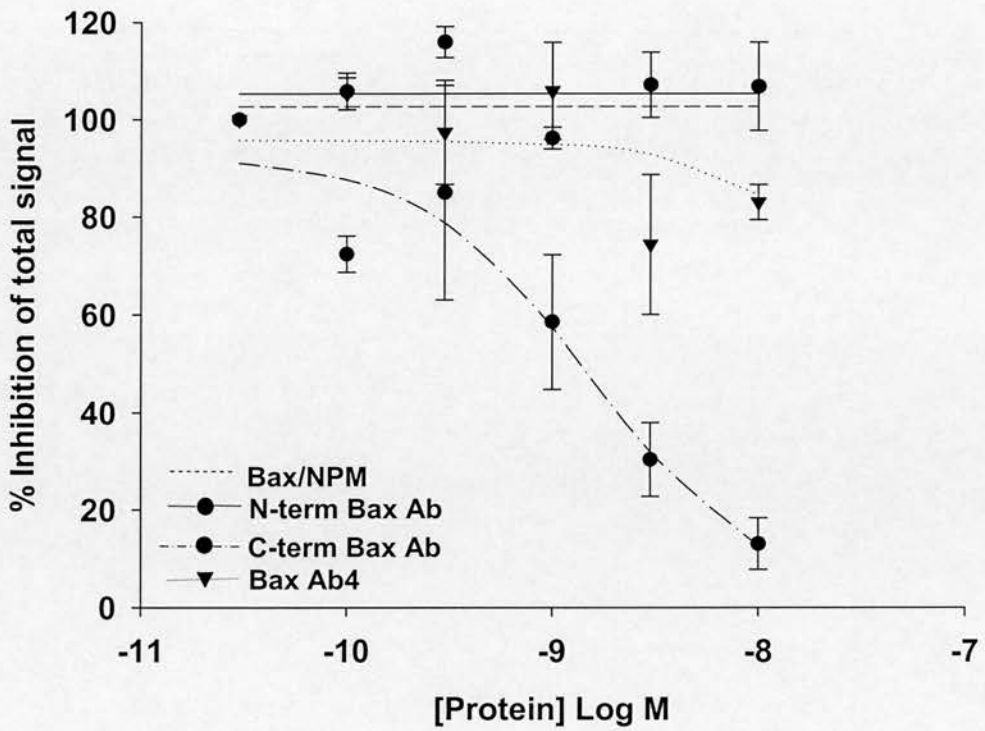
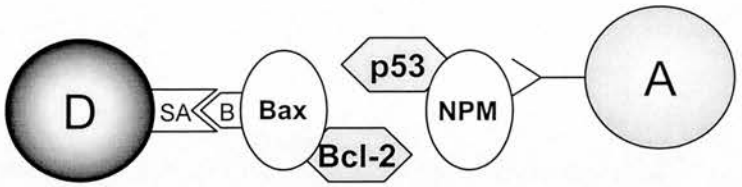


Figure 5.14. Competition studies with Bax-directed antibodies. Recombinant Bax was pre-incubated with antibodies directed against the Bax N-terminus (NT), C-terminus (CT) or α 5 helix (α5) of the pore-forming domain (Bax Ab4) prior to measuring interaction with recombinant nucleophosmin (NPM) using the proximity assay **(a)**. The C-terminal antibody inhibited the Bax-nucleophosmin interaction (IC_{50} = 1.43 $\pm$ 0.65nM), whereas neither the N-terminal nor pore-forming domain-directed antibodies were able to do so **(b)**. A reaction containing no inhibitors was also run (Bax/NPM). Data represent mean $\pm$ s.e.m of three independent experiments.

a.



b.

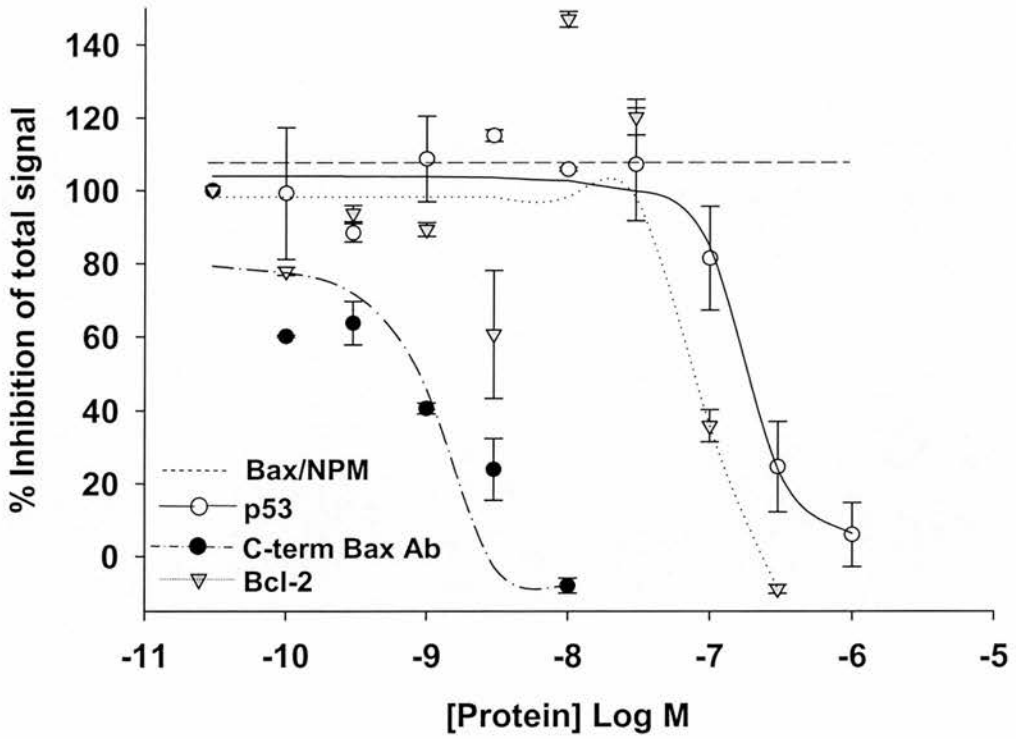


Figure 5.15. Competition studies with putative Bax/nucleophosmin inhibitors. Recombinant Bax and nucleophosmin proteins were pre-incubated with peptides derived from p53 and Bcl-2 prior to mixing and interaction measured using the proximity assay **(a)**. Addition of p53 and Bcl-2 peptides inhibited interaction between Bax and nucleophosmin, with IC50 values of 200±70nM and 100±70nM, respectively **(b)**. Reactions containing Bax C-terminal antibody or without inhibitors were included as positive and negative controls, respectively. Data represent mean ± s.e.m of three independent experiments.

Bax-nucleophosmin interaction measured by proximity assay. Concentration response studies revealed that both the Bcl-2 and p53 peptides inhibited the interaction between Bax and nucleophosmin with apparent IC_{50} values of 100±70nM and 200±70nM, respectively – see Figure 5.15.

5.5. Discussion

This chapter described the design and synthesis of recombinant Bax and nucleophosmin proteins and the analysis of their interaction at a molecular level using an *in vitro* proximity assay.

Although full length human Bax and nucleophosmin ORFs were subcloned into expression plasmids compatible with the expression of recombinant proteins in both prokaryotic and eukaryotic host cells, recombinant proteins were, in the end, produced using *E.coli*-based expression systems. A high yield (milligram quantities) of recombinant nucleophosmin was expressed in BL21(DE3) PlysS *E.coli* cells from the T7 promoter using the pET expression technology, whereas recombinant Bax was expressed from the same plasmid using a cell-free expression system containing *E.coli* extract due to the apparent cytotoxicity of Bax to *E.coli* host cells. Proteins were N-terminally tagged with poly-His to ensure compatibility with down-stream affinity-based purification steps.

Recombinant proteins expressed using eukaryotic translation machinery undergo post-translational modifications, such as disulphide bond formation and *N*-linked glycosylation of membrane-bound or secreted proteins, which ensures correct folding and bioactivity, respectively, within the eukaryotic cell. Prokaryotically-expressed recombinant proteins, however, do not undergo such sophisticated post-translational steps and may therefore exhibit sub-optimal folding and bioactivity compared to endogenous proteins (Swartz, 2001). Furthermore, differences in codon usage between prokaryotes and eukaryotes (particularly the arginine codons AGA and AGG which are rarely found in *E.coli* but are common in eukaryotes) can lead to translational defects when attempting to express eukaryotic proteins in *E.coli* (Baneyx, 1999). However, *E.coli* was used as the host in which the first ever recombinant DNA pharmaceutical was produced, leading to the approval of Eli Lilly's human insulin in 1982, and was also

used to produce Monsanto's bovine growth hormone (bGH) in 1994 (Swartz, 2001). Both of these proteins require extensive post-translational oxidative protein folding steps, and human insulin is a heterodimer, which together testifies to the ability of prokaryotic hosts, such as *E.coli*, to produce complex recombinant eukaryotic proteins with full bioactivity (Swartz, 2001). Nevertheless, to ensure that the prokaryotically-expressed Bax and nucleophosmin proteins were comparable to endogenous human proteins, recombinant proteins were subjected to analysis by SDS-PAGE, Western blot and MALDI-TOF mass spectrometry. From these experiments, Bax and nucleophosmin proteins of anticipated molecular weight (taking into account poly-His tags), that were recognised by specific antibodies raised against human proteins, and that exhibited peptide mass fingerprints matching those of human Bax and nucleophosmin, were produced in sufficient quantity and purity for experimental analysis.

A cell-free amplified luminescent proximity assay was developed to investigate the interaction between recombinant Bax and nucleophosmin proteins at the molecular level. Using this assay, a direct interaction between Bax and nucleophosmin was determined, supporting previous data obtained from the co-immunoprecipitation studies presented in Chapter 4. Affinity chromatography studies, also performed in Chapter 4, identified nucleophosmin as a protein binding specifically to a mutant S184V Bax C-terminal peptide that has been observed to mimic the conformation of this domain in activated Bax (Nechushtan *et al.*, 1999). Due to solubility issues, assessment of the interaction between nucleophosmin and the Bax C-terminal peptides using the proximity assay was not achievable. However, an interaction between recombinant full-length Bax and nucleophosmin proteins using the proximity assay was only observed to occur in the presence of the non-ionic detergent NP40. Exposure of Bax to non-ionic detergents such as NP-40 has been previously demonstrated to induce structural alterations within the Bax N- and C-termini that are representative of those occurring during apoptosis (Hsu & Youle, 1997; 1998). One of these conformational changes, exposure of the 6A7 epitope (residues 12-24) within the Bax N-terminal domain, was confirmed by immunoprecipitating Bax with a conformation-sensitive, 6A7-reactive Bax antibody.

This therefore suggests that nucleophosmin preferentially binds Bax when in its activated, 6A7-reactive, conformation. Such as interaction has been recently reported for another Bax-binding protein, clusterin, although in contrast to the proposed role for nucleophosmin, the Bax-clusterin interaction is thought to be inhibitory (Zhang *et al.*, 2005).

Furthermore, the interaction between Bax and nucleophosmin was disrupted by the addition of an antibody directed against the Bax C-terminus, but not by antibodies directed against αhelix 5 of the pore-forming domain or the N-terminal domain of Bax. These data add further support to the observed binding of nucleophosmin to the Bax C-terminal tail in the affinity chromatography studies performed in Chapter 4. In addition, since the Bax C-terminus is thought to be solvent exposed only in the activated Bax conformation (Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000), this finding also provides an explanation as to why nucleophosmin was observed to only bind to detergent-activated Bax in the proximity assay.

The role played by the Bax C-terminal tail/αhelix 9 in the bioactivity of Bax remains controversial due to conflicting opinions as to its role in the targeting and docking of Bax with the OMM during apoptosis. However, there is a consensus within the literature that the Bax C-terminus functions as a putative membrane-targeting domain to direct the translocation of Bax to the OMM following its exposure as a result of conformational changes occurring within both the N- and C-termini (Goping *et al.*, 1998; Nechushtan *et al.*, 1999; Schinzel *et al.*, 2004b). In this thesis, and in recently published results originating from work within the ACE laboratory (Kerr *et al.*, 2006), nucleophosmin has been demonstrated to bind exclusively to a mutant S184V Bax C-terminal peptide using affinity chromatography (Chapter 4) and, in this chapter, preferentially to the activated Bax conformation using a proximity assay. These data therefore provide further evidence to support the potentially important regulatory contribution made by nucleophosmin to the bioactivity of Bax, as will be investigated in more detail in the following chapter.

Putative binding partners to both Bax and nucleophosmin were included within the proximity assay reaction mix to assess their ability to inhibit the interaction between recombinant proteins. Peptides originating from human Bcl-2 and p53 were chosen in order to identify additional interface domains within the Bax and nucleophosmin protein structures, respectively. Bcl-2 is a known Bax-binding protein (Antonsson *et al.*, 1997; Otter *et al.*, 1998) and was observed in this study to inhibit the interaction between Bax and nucleophosmin with an apparent IC_{50} of 100±70nM. Heterodimerisation between opposing members of the Bcl-2 protein is a central mechanism by which this protein family regulates apoptosis, and has been demonstrated to involve the amphipathic BH3 domain (Sattler *et al.*, 1997). Indeed, the Bax BH3 domain has been shown to be essential for its interaction with Bcl-2 (Zha *et al.*, 1996). However, confirmation that the BH3 domain on Bax forms part of the interface with nucleophosmin was not achievable since a Bax BH3 domain-reactive antibody could not be sourced to validate this result. Consequently, it cannot be ruled out that the blocking effect observed using the Bcl-2 peptide could be due to steric hindrance with another distinct binding site on Bax.

Addition of a p53 peptide to the proximity assay reaction mix also inhibited interaction between Bax and nucleophosmin with an apparent IC_{50} of 200±70nM. The interaction between nucleophosmin and p53 has been previously shown to involve the C-terminal heterodimerisation domain within nucleophosmin (Colombo *et al.*, 2002; Li *et al.*, 2005). Recent biochemical analyses of this interaction have pin-pointed the p53-binding motif to lie within residues 249-262 of nucleophosmin (Lambert and Buckle, 2006). Unfortunately, the p53-binding site within nucleophosmin overlaps with the immunizing peptide (residues 226-294) used to raise the monoclonal antibody by which nucleophosmin is attached to the acceptor beads in the proximity assay. This therefore casts doubt on the validity of the observed inhibitory effect attributed to the p53 peptide, as it is possible that the p53 peptide competed with the nucleophosmin C-terminal domain for binding to the acceptor beads instead of inhibiting the Bax-nucleophosmin interaction. Furthermore, since nucleophosmin is attached to the acceptor beads in the

proximity assay via its C-terminus, the resulting data suggest that it is the N-terminal half of nucleophosmin that holds the Bax-interaction domain. The N-terminus of nucleophosmin contains several hydrophobic regions that have together been attributed with holding its chaperoning capability (Hingorani *et al.*, 2000). This finding is therefore consistent with the hypothesis, as set out in this thesis, that nucleophosmin functions as a molecular chaperone to Bax during its translocation to the mitochondrial surface in apoptotic cells.

For the purposes of this thesis, the proximity assay was used as a tool to characterise the direct interaction between recombinant Bax and nucleophosmin proteins. However, this technology can also be used as a platform to conduct HTS assays to identify small-molecule inhibitors/enhancers of a particular protein-protein interaction. The reliability of this 384-well assay is reflected by a signal to noise ratio of 135, an inter-plate variance of 14.8% (across days) and a Z' value of 0.50 which puts this assay at the cusp between marginal and excellent as a high-throughput screen (Zhang *et al.*, 1999). Although out-with the scope of this thesis, a manual screen of a small chemical library (800 compounds) was performed using the proximity assay by researchers within ACE to identify pharmacological modulators of the Bax-nucleophosmin interaction. From these preliminary studies, 17 potential compounds were identified that modulated Bax peptide-nucleophosmin binding either by inhibiting or enhancing the signal by 50%. One hit compound, 2-(5-methyl-2-phenyl-1,3-thiazol-4-yl)ethanohydrazide, produced a concentration-dependent partial inhibition of the Bax-nucleophosmin interaction with an IC_{50} value of 100nM. Furthermore, this compound was observed to confer a degree of protection to SH-SY5Y cells against UV-irradiation when administered into culture medium, suggesting that nucleophosmin may be involved in regulating Bax bioactivity in response to different apoptotic stimuli (Thompson *et al.*, manuscript submitted to journal).

5.6. Summary

Recombinant full-length human Bax and nucleophosmin proteins were successfully produced using prokaryotic-based expression systems to enable analysis of the Bax-nucleophosmin interaction in a cell-free proximity assay. An *in vitro* amplified luminescent proximity assay was established and optimised to investigate the Bax-nucleophosmin interaction at a molecular level. Using this assay, three aspects of the Bax-nucleophosmin interaction were determined: 1) The observed binding kinetics of the Bax-nucleophosmin interaction are indicative of a competitive and specific biological interaction, 2) the interaction between Bax and nucleophosmin involves the Bax C-terminal domain and the N-terminal half of nucleophosmin, and 3) nucleophosmin only binds to the detergent-activated Bax conformation, which suggests that nucleophosmin interacts with Bax during apoptosis.

CHAPTER 6: Characterising the biological role of nucleophosmin in the intrinsic apoptotic pathway

6.1 Chapter aims

The aim of this chapter is to determine the role played by nucleophosmin, as a molecular chaperone to Bax, within the intrinsic apoptotic pathway. To achieve this, RNA interference will be employed to attenuate nucleophosmin protein expression and the impact this has on the progression of the intrinsic apoptotic pathway assessed using the neuronal cell model established in Chapter 3.

6.2 Introduction

6.2.1 Nucleophosmin

Nucleophosmin (NPM, also known as B23, numatrin and NO38) is a multifunctional phosphoprotein localised within the nucleolus of most, if not all, cells – see Figure 6.1. Nucleophosmin was first characterised as a ribosomal RNA assembly and transport factor in the nucleolus (Olson *et al.*, 1986), and has since been attributed with nucleic acid binding, ribonuclease and molecular chaperone activities (Hingorani *et al.*, 2000). However, recent studies have identified an increasingly observed role for nucleophosmin in the molecular pathways governing cellular proliferation, oncogenesis and apoptosis (Colombo *et al.*, 2002; Maiguel *et al.*, 2004; Li *et al.*, 2005; Ahn *et al.*, 2005; Grisendi *et al.*, 2005b).

6.2.2 Nucleophosmin protein structure

Nucleophosmin belongs to the nucleoplasmin (Np) family of nuclear chaperones (Dingwall and Laskey, 1990), the archetypal member of which was the first molecular chaperone to be described (Laskey *et al.*, 1978). The nucleophosmin polypeptide chain

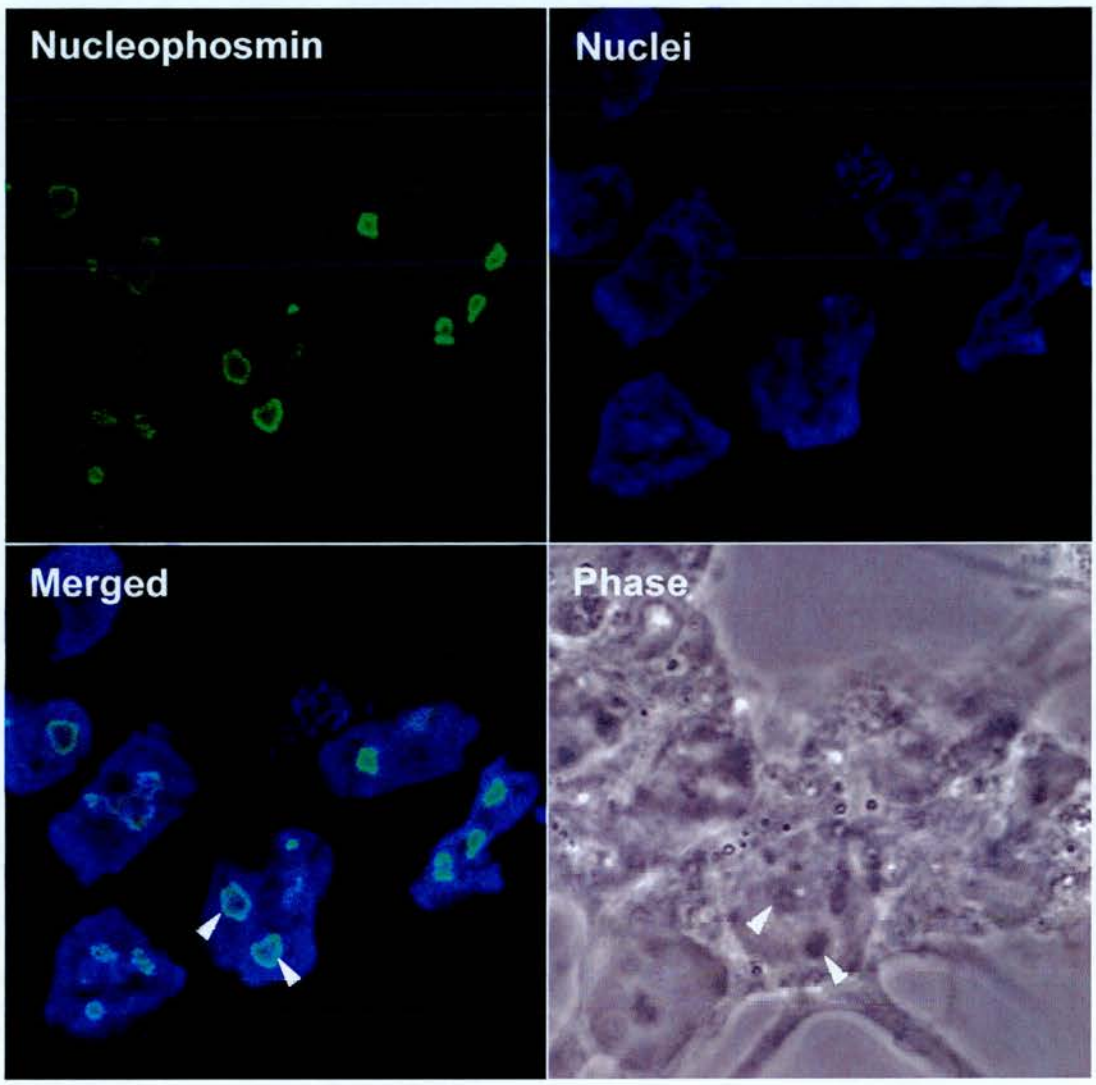


Figure 6.1. Nucleolar localisation of nucleophosmin. Nucleophosmin (green) was identified as a protein within nucleoli (indicated by arrows) of cultured SH-SY5Y cells using confocal microscopy. Nuclei are shown in blue (TO-PRO-3).

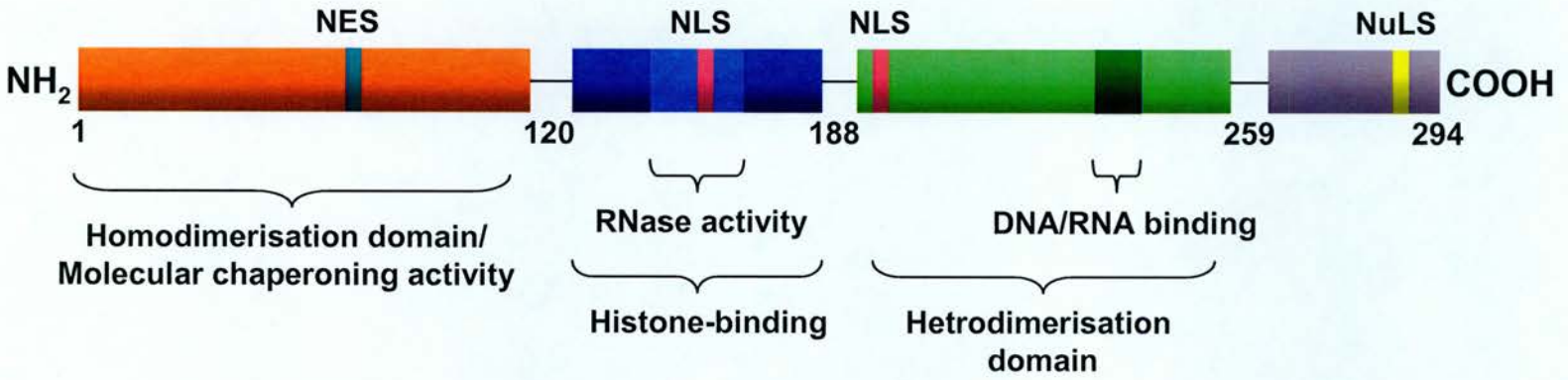


Figure 6.2. Nucleophosmin functional domains. The N-terminus contains the hydrophobic homodimerisation domain involved in molecular chaperoning and nuclear export signal (NES) motif (residues 94-102). The central acidic region is important for histone binding and holds nucleophosmin's endoribonuclease activity. The heterodimerisation domain within the C-terminal half is involved in interactions with p53, p120 and HIV Tat proteins and also contains a basic region involved in nucleic acid binding. The B23.2 isoform lacks the final 32 amino acids (259-294) that contains the nucleolar localisation signal (NuLS) motif (residues 288-290). Two nuclear localisation signal (NLS) motifs have been mapped to residues 152-157 & 190-197. Data compiled from Hingorani *et al.*, 2000.

exhibits a highly modular structure, containing distinct sequence motifs arranged into several functional domains, which together account for the diverse biochemical functions of this protein (Hingorani *et al.*, 2000) – see Figure 6.2 for an overview of the nucleophosmin protein structure. As is common to many molecular chaperones, nucleophosmin oligomerises into hexamers and larger complexes under native conditions within the nucleolus (Schmidt-Zachmann *et al.*, 1987; Herrera *et al.*, 1996) which enhances its ability to dispel large protein aggregates. In this multimeric form, nucleophosmin is highly stable with a cellular half life of more than 24h (Itahana *et al.*, 2003).

Two splice variants of nucleophosmin are expressed in mammalian tissues: B23.1 and the C-terminal-truncated isoform, B23.2. B23.2 lacks the C-terminal nucleolar localisation signal and is therefore distributed within the nucleoplasm in addition to the nucleolus (Savkur and Olson, 1998). B23.1, however, is the predominantly expressed isoform in all tissues (Wang *et al.*, 1993).

6.2.3 Nucleophosmin "house-keeping" roles

As a multi-functional protein, nucleophosmin engages a diverse range of proteins during the course of its cellular activities – see Table 6.1. Within the nucleolus, nucleophosmin performs essential functions as a molecular chaperone (Szebeni and Olson, 1999) and in the assembly of pre-ribosomal subunits (Savkur and Olson, 1998). As a molecular chaperone, nucleophosmin counteracts protein aggregation within the crowded confines of the nucleolus and aids the transport of numerous proteins across the nucleolar membrane, including the transcription factor YY1 (Inouye and Seto, 1994), the tumour suppressor protein, $p19^{ARF}$ (Korgaonkar *et al.*, 2005), the retinoblastoma protein (pRb) (Takemura *et al.*, 2002) and several viral proteins, including HIV Rev and Tat proteins (Fankhauser *et al.*, 1991; Li, 1997). Using immunocytochemistry, nucleophosmin has been localised to the peripheral regions of the dense fibrillar component (DFC) and granular component (GC) of the nucleolus (Spector *et al.*, 1984; Biggiogera *et al.*,

NPM interacting protein	Sucellular localisation	Function of interaction	References
Nucleolin	nucleolus	Partner protein in ribosomal biogenesis	1
p120	nucleolus	Unknown	2
YY1	nucleus	Reverses YY1-induced transcriptional repression	3
PKR	nucleus	Inhibits PKR tumour suppressor activity	4
ARF	nucleolus	Supresses RNA synthesis & cell proliferation	5, 6, 7, 8, 9
MDM2	nucleolus/nucleus	MDM2 down-regulation & p53 stability	10
p53	nucleus	Promotes p53 transcriptional activity	11
pRb	nucleus	pRb nucleolar translocation	12
Gadd45a	nucleus	Gadd45a nuclear translocation	13
PARP I & II	nucleolus/nucleus	Possible role in DNA repair response	14
Caspas-3	nucleus	Caspase-3 substrate target	15
CAD/DFF40	nucleus	Inhibits DNase activity	16
HIV Rev & Tat	nucleus	Rev & Tat nuclear translocation	17, 18
Kaposi's sarcoma virus K cyclin	nucleus	NPM nuclear translocation & p53 stabilisation	19
Hepatitis delta antigens	nucleus	Promotes viral RNA replication	20
Japanese encephalitis virus core protein	nucleus/cytoplasm	Promotes viral RNA replication	21

Table 6.1. Known nucleophosmin-binding proteins. References: **1.** Liu *et al.*, 1999. **2.** Li *et al.*, 1996. **3.** Inouye & Seto, 1994. **4.** Pang *et al.*, 2003. **5.** Itahana *et al*, 2003. **6.** Bertwistle *et al.*, 2004. **7.** Brady *et al.*, 2004. **8.** Korgonkar *et al.*, 2005. **9.** Colombo *et al.*, 2005. **10.** Kurki *et al.*, 2004. **11.** Colombo *et al.*, 2002. **12.** Takemura *et al.*, 2002. **13.** Meder *et al.*, 2005. **14.** Gao *et al.*, 2005. **15.** Chou & Yung, 2001. **16.** Ahn *et al.*, 2005. **17.** Fankhauser *et al.*, 1991. **18.** Li, 1997. **19.** Kurki *et al.*, 2004. **20.** Huang *et al.*, 2001. **21.** Tsuda *et al.*, 2006.

1990), in which the maturation of pre-ribosomal RNAs is thought to take place (Fakan and Bernhard, 1971). Utilising its intrinsic endoribonuclease activity (Herrera *et al.*, 1995), nucleophosmin has been proposed to assist in the processing of ribosomal RNA (rRNA) precursors at these sites into mature rRNA subunits prior to their export into the nucleus (Savkur and Olson, 1998).

6.2.4 The role of nucleophosmin in development

In 2005, two landmark studies characterising the resulting phenotype of nucleophosmin gene knockout mice demonstrated the crucial role played by nucleophosmin in embryonic development (Colombo *et al.*, 2005; Grisendi *et al.*, 2005b). Homozygous mutant offspring (*Npm1*$^{-/-}$) suffered extensive developmental defects and death by embryonic day (E) 12.5. Even *Npm1* hypomorphic mutants (*Npm1*$^{hy/hy}$), where nucleophosmin expression was reduced to about 10-30% of normal levels, died during embryogenesis, albeit at a later stage (E16.5) (Grisendi *et al.*, 2005b). Immunohistochemical analysis of normal embryos demonstrated high and widespread expression of nucleophosmin during this developmental period (Colombo *et al.*, 2005). The most visible defects arising from this genetic manipulation were in the organogenesis of liver and forebrain (specifically the telencephalic region and eye) and impaired development and subsequent differentiation of haematopoietic precursors leading to a compromised foetal blood supply and severe anaemia (Grisendi *et al.*, 2005b). This latter finding is in agreement with another recent report demonstrating nucleophosmin's requirement for haematopoietic stem cell expansion and differentiation, as well as the protection of haematopoietic progenitor cells against stress-induced DNA-damage (Li *et al.*, 2006). Furthermore, the extensive DNA damage and genomic instability observed in the nucleophosmin knock-out animals is in agreement with earlier studies implicating nucleophosmin as a major DNA-damage response protein (Wu *et al.*, 2002; Meder *et al.*, 2005), and the unrestricted centrosome duplication and multiple mitotic spindle formation observed in Npm1$^{-/-}$ MEFs supports

the proposed role for nucleophosmin as an essential mediator of CDK2/cyclin E-directed centrosome duplication during mitosis (Okuda, 2002).

6.2.5 Nucleophosmin and cancer

That nucleophosmin may play an important role in cell growth and oncogenesis was realised by early observations that its cellular levels increased rapidly in actively proliferating and malignant cells (Chan *et al.*, 1989). Conversely, nucleophosmin expression was observed to be down-regulated in cells undergoing differentiation (van Belzen *et al.*, 1995) or apoptosis (Patterson *et al.*, 1995). Furthermore, the involvement of nucleophosmin in several oncogene-forming gene translocations has made it a key marker protein for several human haematopoietic malignancies (Grisendi *et al.*, 2005b) – see Table 6.2. A well characterised gene translocation involving nucleophosmin underlies a large proportion of cases of anaplastic large cell lymphoma (ALCL), an aggressive T-cell lymphoma most commonly seen in children (Grisendi *et al.*, 2005a). In this gene translocation (2;5)(p23;q35), the *Npm1* gene (chromosomal locus 5q35) becomes fused with the anaplastic lymphoma kinase (ALK) gene (locus 2p23) and encodes a constitutively activated tyrosine kinase fusion gene product, NPM-ALK, which has been proposed to function as an oncogene in part through phosphorylation/activation of the anti-apoptotic protein kinase Akt (Rassidakis *et al.*, 2005). Aside form ALCL, translocation of *Npm1* to additional fusion partners, as well as *Npm1* gene mutations, underlies several other human haematological malignancies, including promyelocytic leukaemia, acute myeloid leukaemia and chronic myelogenous leukaemia (Grisendi *et al.*, 2006).

A role for nucleophosmin in perturbing progression of the cell cycle in response to oncogenic cues and genotoxic stress has also been suggested through its observed binding to several key regulatory proteins of the cell cycle. In response to genotoxic stress and hyperproliferative signals, such as those directed by *c-myc*, the tumour suppressor protein, $p19^{ARF}$, has been shown to interact functionally with nucleophosmin

NPM gene alteration	Mutated product	Resultant malignancies	References
Overexpression	Increased protein levels	Various solid tumours: gastric, ovarian, colon & prostate carinomas	1
Translocation: t(5;17)(q35;q12)	NPM-RARα	Acute promyelocytic leukemia	2
Translocation: t(2;5)(p23;q35)	NPM-ALK	Anaplastic large cell lymphoma	3
Translocation: t(3;5)(q25;q35)	NPM-MLF1	Myelodysplastic syndrome & acute myeloid leukemia	4
Mutation (exon 12)	NPMc+ (cytoplasmic mutant protein)	Acute myeloid leukemia with a normal karyotype	5
Deletion (-5q35, -5)	No expression	non-small-cell lung carcinoma	6

Table 6.2. Genetic basis underlying the involvement of nucleophosmin in human cancer. NPM over-expression is a marker for various tumours, including gastric, colon, ovarian and prostate carcinomas. In addition, NPM is one of the most frequently altered genes in haematopoietic tumours. References: **1.** Grisendi *et al.*, 2006. **2.** Redner *et al.*, 1996. **3.** Morris *et al.*, 1994. **4.** Yoneda-Kato *et al.*, 1996. **5.** Falini *et al.*, 2005. **6.** Mendes-da-Silva *et al.*, 2000. Table adapted from Grisendi *et al.*, 2006.

to retard ribosome biogenesis and induce cell cycle arrest (Itahana *et al.*, 2003; Bertwistle *et al.*, 2004; Brady *et al.*, 2004; Korgaonkar *et al.*, 2005). Reported interaction between nucleophosmin and the tumour suppressor protein, p53, and its inhibitor, MDM2, has also been shown to induce cell cycle arrest in response to genotoxic stress (Colombo *et al.*, 2002; Kurki *et al.*, 2004). Finally, induction of cell cycle arrest by two further cell cycle-regulatory proteins, the tumour suppressor protein, retinoblastoma protein (pRb) and the transcription factor, Gadd45a, has also been shown to involve nucleophosmin (Takemura *et al.*, 2002; Gao *et al.*, 2005). In both cases, nucleophosmin has been observed to chaperone a nuclear translocation event late in the cell cycle that is thought to be required for pRb and Gadd45 to induce cell cycle arrest at the G2/M boundary.

6.2.6 Nucleophosmin and apoptosis

When this thesis was proposed, a functional involvement of nucleophosmin in positively regulating the stability and transcriptional activity of the tumour suppressor protein, p53, had recently been reported (Colombo *et al.*, 2002). As a consequence of this interaction, nucleophosmin was implicated as a facilitator of p53-depedant cell-cycle arrest and apoptosis in response to genotoxic stress. However, this report conflicted with another study in which the attenuation of nucleophosmin protein expression by anti-sense treatment was observed to increase the sensitivity of cells to p53-dependent apoptosis induced by exposure to UV irradiation (Wu *et al.*, 2002), suggesting that nucleophosmin normally protects cells against genotoxic stresses by suppressing p53 activity. Furthermore, nucleophosmin had also been identified as a nuclear substrate of caspase-3 during the execution phase of apoptosis (Chou and Yung, 2001). The role of nucleophosmin in apoptosis at the time when this thesis was proposed was therefore somewhat ambiguous. However, its reported interaction with p53 does suggest that nucleophosmin could be an important regulatory protein in genotoxic stress-induced apoptosis. An identifiable role for nucleophosmin in additional apoptotic pathways or a functional interaction with apoptogenic proteins out-with the nucleus, however, has to

date not been demonstrated. Thus, as described within this thesis, the identification of nucleophosmin as a novel Bax-binding protein is an exciting and novel finding, and reveals a potential extra-nuclear function for nucleophosmin in the regulation of apoptosis and cell proliferation.

6.3 Materials & Methods

6.3.1 Cell culture

SH-SY5Y cells were grown and maintained as described in general materials & methods (Chapter 2). Seeding densities, culture vessels and protocols used were optimised for each method as described below.

6.3.2 Co-immunoprecipitation

Co-immunoprecipitation was performed as described in general materials & methods (Chapter 2). Briefly, SH-SY5Y whole cell lysates were immunoprecipitated with 5μg of either a pan-reactive polyclonal Bax antibody (Upstate Biotechnology), a monoclonal nucleophosmin antibody (Sigma) or a monoclonal SMN antibody (BD Biosciences) and co-immunoprecipitants collected with protein A-conjugated dynabeads (Invitrogen). Proteins were then separated by SDS-PAGE and analysed by Western blot using antibodies against Bax, nucleophosmin and GRB2, as described in general materials & methods (Chapter 2).

6.3.3 Confocal microscopy

Confocal microscopy was used to investigate the translocation of Bax and nucleophosmin proteins during exposure to STS in SH-SY5Y cells as described in general materials & methods (Chapter 2). Briefly, DMSO vehicle-treated (Control) cells and cells exposed to 2h or 4h STS were fixed and probed with antibodies against Bax (Upstate Biotechnology) or nucleophosmin and stained with fluorescent secondary antibodies (AlexaFluor-488 and -546, respectively) before analysis by confocal microscopy. Co-localisation experiments were also attempted but were unsuccessful

due to incompatible fixing procedures for the Bax and nucleophosmin antibodies available.

6.3.4 RNA interference

Three different methodologies were used to employ RNA interference (RNAi) against endogenous human nucleophosmin: 1) Four hairpin siRNA (shRNA) sequences expressed from a plasmid DNA vector back-bone (pSilencer; Ambion, Warrington, UK), 2) a single published small interfering RNA (siRNA) oligonucleotide (Colombo *et al.*, 2002), and 3) Dharmacon's siGenome SMARTpool™ siRNA consisting of four individual siRNA oligonucleotides combined within a single solution (Dharmacon Inc., Lafayette, CO, USA).

6.3.5 Nucleophosmin shRNA expression vector design and manufacture

Complementary oligonucleotide sequences were designed for cloning into shRNA expression vectors containing the RNA polymerase III promoters U6 (pSilencer 2.1-U6 hygro) or H1 (pSilencer 3.1-H1 hygro; both from Ambion). Four target sequences meeting the suggested criteria (21 nucleotides long, G/C content 40-55%, not containing a run of more than 4 or 5 As or Ts (these act as termination sites for RNA polymerase III), and spread along the length of the cDNA sequence (Reynolds *et al.*, 2004)) were chosen from within nucleophosmin cDNA open reading frame (ORF) using an online siRNA target finder and design tool (http://www.ambion.com/techlib/misc/siRNA_finder.html) – see Table 6.3 for sequence information. The specificity of each sequence for nucleophosmin mRNA was confirmed using the on-line Basic Local Alignment Search Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST/). Oligonucleotides were synthesised (Invitrogen) and complementary sequences annealed and cloned into each of the pSilencer vectors – see Figure 6.3 for an overview of this procedure. pSilencer plasmid DNA was then transformed into competent DH5α cells (Invitrogen) and Ampicillin-

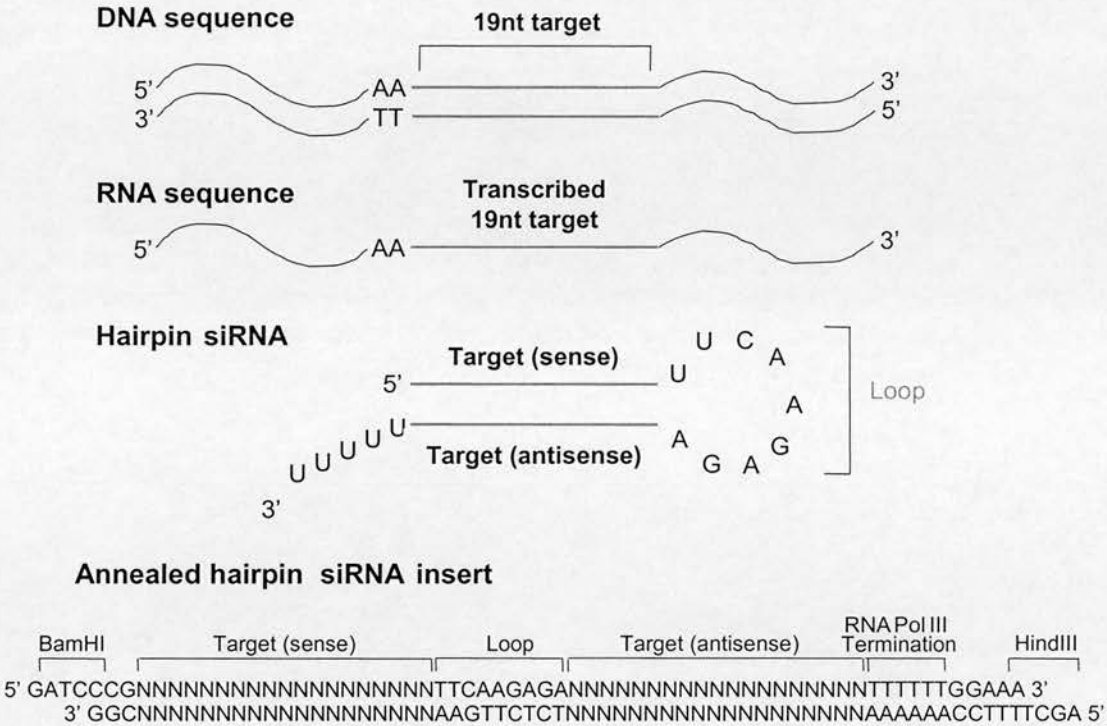


Figure 6.3. Design of shRNA expression cassette for cloning into pSilencer expression vectors. For each 19-nucleotide target sequence, complementary 55-60mer oligonucleotides, with 5' single-stranded restriction enzyme site overhangs, were designed. The two complementary domains of the 19-mer hairpin target sequences were separated by a loop sequence and the 3' end contained a poly-thymidine tract to terminate RNA polymerase III-mediated transcription. Downstream of the RNA terminator site, a short 5'-GGAA-3' sequence was included for optimal gene silencing. The 5' ends of the two oligonucleotides were non-complementary and included BamHI and HindIII restriction overhangs to facilitate efficient directional cloning into the pSilencer vectors. A guanosine residue was included immediately downstream of the BamH1 site because RNA polymerase III transcription is more efficient when initiated at a purine.

resistant cells containing recombinant plasmid DNA were selected and plasmid DNA prepared according to the manufacturer's instructions (Wizard Plus SV minipreps; Promega). Cloned sequences were verified by DNA sequencing (DNA Sequencing Facility, Department of Biochemistry, University of Oxford) and restriction digestion using insert sequence-specific cutting enzymes.

6.3.6 Nucleophosmin siRNA design

Nucleophosmin-specific siRNA oligonucleotides were designed from published sequences targeting nucleotides 103-125 of human nucleophosmin cDNA relative to the first nucleotide of the start codon (Colombo *et al.*, 2002). The oligonucleotide sequences were as follows: sense: 5'-UGAUGAAAAUGAGCACCAGTT-3'; antisense: 5'-CUGGUGCUCAUUUUCAUCATT-3'. An inverted sequence was used as a control siRNA (sense: 5'-GACCACCAGUAAAAGUAGUTT-3'; antisense: 5'-ACUACUUUUACUCGUGGUCTT-3'). All oligonucleotides were synthesised by Invitrogen and annealed on arrival.

siRNAs targeting human nucleophosmin were also obtained from Dharmacon Inc. as SMARTpool™ ready-to-transfect reagents containing four individual siRNAs (sequence information unavailable). Lyophilised siRNAs were reconstituted in provided buffer to a final concentration of 20μM and stored at -20°C.

6.3.7 Transfection of shRNA and siRNA silencing complexes

To maximise transfection efficiency of pSilencer shRNA plasmid DNA vectors into SH-SY5Y cells, optimisation experiments were performed using a hand-held gene gun (Helios Gene-gun; Bio-rad Laboratories) or a cationic lipid-based transfection reagent (Lipofectamine 2000; Invitrogen) to transfect cells with plasmid DNA containing the reporter gene Yellow Fluorescent Protein (YFP) (pZsYellow1-N1; BD Biosciences).

Human NPM target sequence	Oligonucleotide	Oligonucleotide sequence (5'-3')
Nucleotides 182-202: AAG CAG AGG CAA TGA ATT ACG	Sense	AGC TTT TCC AAA A**AA GCA GAG GCA ATG AAT TAC G**TC TCT TGA ACG TAA TTC ATT GCC TCT GCG G
	Anti-sense	GAT CCC GCA GAG GCA ATG AAT TAC GTT CAA GAG A**CG TAA TTC ATT GCC TCT GCT T**TT TTG GAA A
Nucleotides 362-282: AAG ATG CAG AGT CAG AAG ATG	Sense	AGC TTT TCC AAA A**AA GAT GCA GAG TCA GAA GAT G**TC TCT TGA ACG TAA TTC ATT GCC TCT GCG G
	Anti-sense	GAT CCC GCA GAG GCA ATG AAT TAC GTT CAA GAG A**CA TCT TCT GAC TCT GCA TCT T**TT TTG GAA A
Nucleotides 470-490: AAC TTC CTG CTG ATG AAG ATG	Sense	AGC TTT TCC AAA A**AA CTT CCT GCT GAT GAA GAT G**TC TCT TGA ACG TAA TTC ATT GCC TCT GCG G
	Anti-sense	GAT CCC GCA GAG GCA ATG AAT TAC GTT CAA GAG A**CA TCT TCA TCA GCA GCA AGT T**TT TTG GAA A
Nucleotides 716-736: AAG GAC CTA GTT CTG TAG AAG	Sense	AGC TTT TCC AAA A**AA GGA CCT AGT TCT GTA GAA G**TC TCT TGA ACG TAA TTC ATT GCC TCT GCG G
	Anti-sense	GAT CCC GCA GAG GCA ATG AAT TAC GTT CAA GAG A**CT TCT ACA GAA CTA GGT CCT T**TT TTG GAA A

Table 6.3. Details of sequences used to construct nucleophosmin shRNA expression vectors. Target sequences within human nucleophosmin cDNA are provided in the left hand column. For each target sequence, a sense and antisense 64-mer hairpin template was designed containing the 21-mer target sequence as its stem (in bold) separated by a 9 nucleotide loop sequence. A 5-6 nucleotide poly(A) tract and a 5' overhang forming the BamHI and HindIII restriction sites were also included.

For the gene gun-based transfection studies, SH-SY5Y cells were seeded at a density of $1x10^6$ cells in 35/22 mm diameter glass-bottomed poly L-lysine coated petri dishes (Willco Wells) and left for 24h to attach. 1μm gold particles were coated with pZsYellow1-N1 according to the manufacturer's instructions. Briefly, 20mg gold particles were suspended in 100μl 0.05M spermidine and 20μg of pZsYellow1-N1. The gold particles were precipitated with 100μl of 1M $CaCl_2$, washed three times in 100% ethanol, re-suspended in 1.2ml 100% ethanol and loaded into Teflon tubing. Medium was removed from wells just before transfection. pZsYellow1-N1-coated gold particles were shot into the cells with the gun pressurized at 150psi or 250psi. Each well received 1mg gold particles containing 1μg pZsYellow1-N1. A 40μm nylon mesh was placed between the gun and culture to protect cells from the shock wave. Cells were replenished with fresh medium after 6h and incubated for 48h before fixing and imaging by confocal microscopy as described in general materials & methods (Chapter 2). Three 20X fields were imaged in each cover slip by confocal microscopy to provide an average value. Transfection efficiency was calculated by counting the proportion of YFP-labelled cells within each field area.

For the cationic lipid-based transfection studies, SH-SY5Y cells were seeded at a density of 1×10^5 in 24-well culture plates. Ratios of plasmid DNA (μg) to transfection reagent (μl) of 1:1, 1:2 and 1:3 were combined according to the manufacturer's instructions. Briefly, plasmid DNA and Lipofectamine 2000 were diluted separately in Optimem reduced serum medium and incubated at room temperature for 5mins before gently combined. Plasmid DNA/lipid solutions were then left for 20mins at room temperature to encourage complex formation before 100μl was pipetted into each well. Cells were transfected for 4h before complexes were replaced with fresh culture medium and cells incubate a further 44h before fixing and analyses by confocal microscopy as described above. siRNA oligonucleotides were transfected using Lipofectamine 2000 according to the manufacturer's instructions.

For nucleophosmin knock-down experiments, pSilencer shRNA vectors were transfected separately or combined to form "pools" into SH-SY5Y cells using Lipofectamine 2000 transfection reagent.

6.3.8 Assessing knock-down of target protein expression

Attenuation of target protein expression was assessed by quantitative Western blot as described in general materials & methods (Chapter 2). Optimisation of siRNA concentration (30nM-300nM) delivered into cells and duration of silencing effect (24h-72h) post transfection was performed. Specificity of RNAi-mediated knockdown of target protein expression was assessed in each experiment by transfecting wells with transfection reagent alone (vehicle) and with control shRNA vectors or siRNA duplexes (control siRNA). The control target sequences used were either scrambled sequences with no known homology to any mammalian mRNA sequence (shRNA experiments) or the target sequence orientated in the opposite direction (siRNA experiments).

6.3.9 Assessment of cytochrome c release, caspase-3 activity, DNA fragmentation and cell viability

Progression of the intrinsic apoptotic pathway in siRNA-treated, control siRNA-treated and transfection alone-treated wells was assessed by measuring cytochrome c release, caspase-3 activity, DNA fragmentation and cell viability as described in Chapter 3.

6.4 Results

6.4.1. Endogenous Bax and nucleophosmin proteins interact

Interaction between endogenous Bax and nucleophosmin proteins in SH-SY5Y whole cell lysates was confirmed using reciprocal co-immunoprecipitation - see Figure 6.4.

6.4.2. Bax and nucleophosmin undergo intracellular translocation during STS-induced apoptosis in SH-SY5Y cells

The sub-cellular localisation of endogenous Bax and nucleophosmin within SH-SY5Y cells during exposure to STS was analysed using fluorescent antibodies and confocal microscopy. During exposure to STS, nucleophosmin underwent a marked re-distribution in its subcellular localisation – see Figure 6.5. In control cells, nucleophosmin was predominantly nucleolar. However, by 2h exposure to STS, nucleophosmin had undergone translocation into the cytosol and remained cytosolic at 4h. In control cells, Bax staining was diffuse and cytosolic but an increasingly punctate staining pattern indicative of mitochondrial association was evident by 2h exposure to STS, becoming marked by 4h.

6.4.3. Attenuating expression of endogenous nucleophosmin protein in SH-SY5Y cells using RNAi

Attempts were initially made to silence endogenous nucleophosmin expression SH-SY5Y cells using a published siRNA sequence targeting nucleotides 103-135 that had been successfully used to silence nucleophosmin expression by up to 60-70% in a U20S cell line (Colombo *et al.*, 2002). In our hands, however, transfection of this siRNA oligonucleotide into SH-SY5Y cells was ineffective at silencing nucleophosmin protein expression – see Figure 6.6. Correspondance with Dr Colombo established that only one

Figure 6.4. Confirming interaction between endogenous Bax-nucleophosmin proteins by recipricol co-immunopreciptation and Western blot. Nucleophosmin was confirmed as a protein co-precipitating with Bax in SH-SY5Y whole cell lysates **(a)**. A control co-immunoprecipitation for the unrelated protein, growth factor-related binding protein 2 (GRB2), was included to demonstrate specificity. Additional confirmation of the Bax-nucleophosmin interaction was demonstrated by reciprocal immunoprecipitation with a nucleophosmin antibody and identification of Bax in the immunoprecipitate **(b)**. Specificity of this interaction was demonstrated by immunoprecipitation of an unrelated protein, survival motor neuron protein (SMN). Detection of proteins by Western blot (WB) in whole cell lysate (lysate) and immunoprecipitate (IP) are as indicated. Data shown are representative of at least three independent experiments.

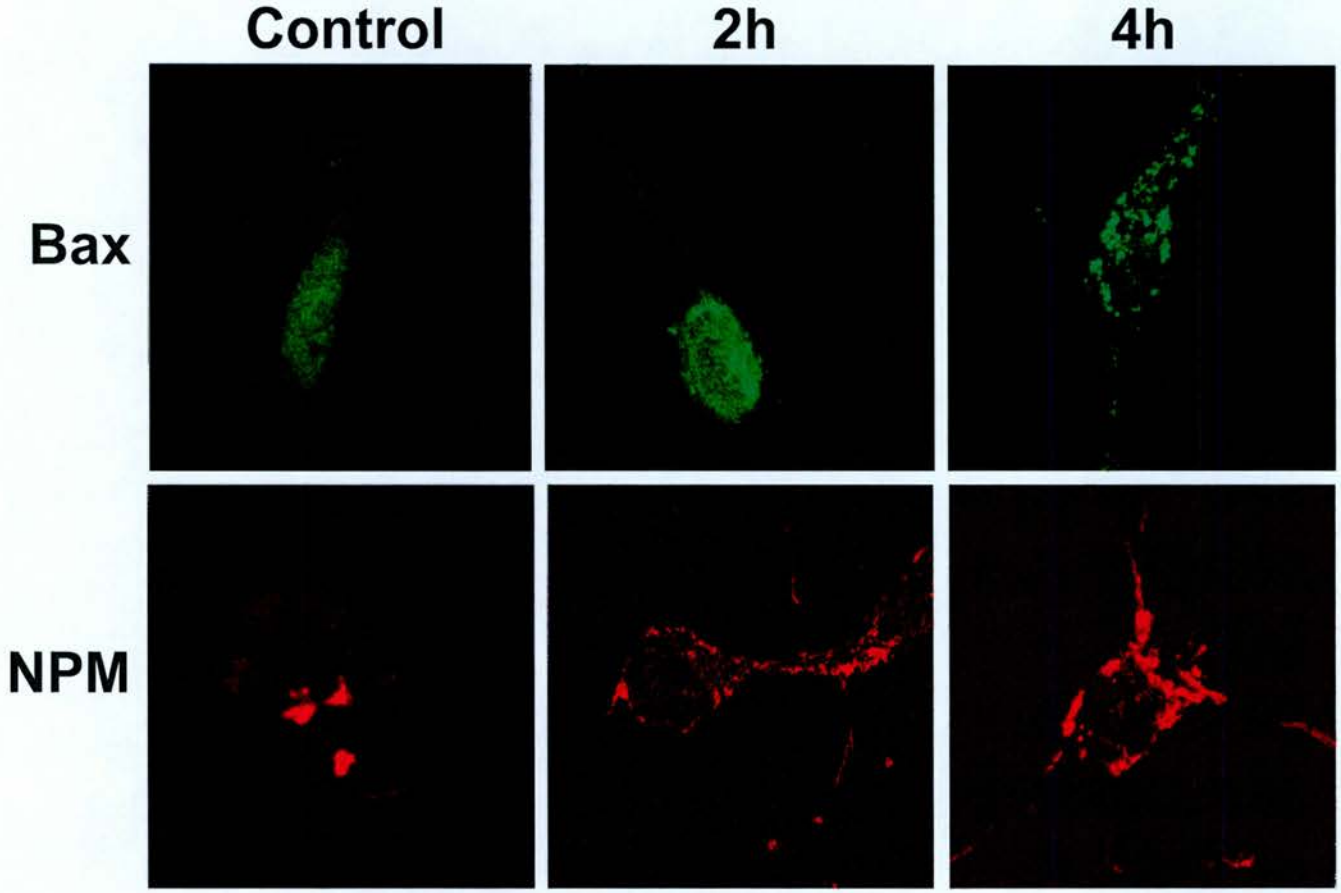
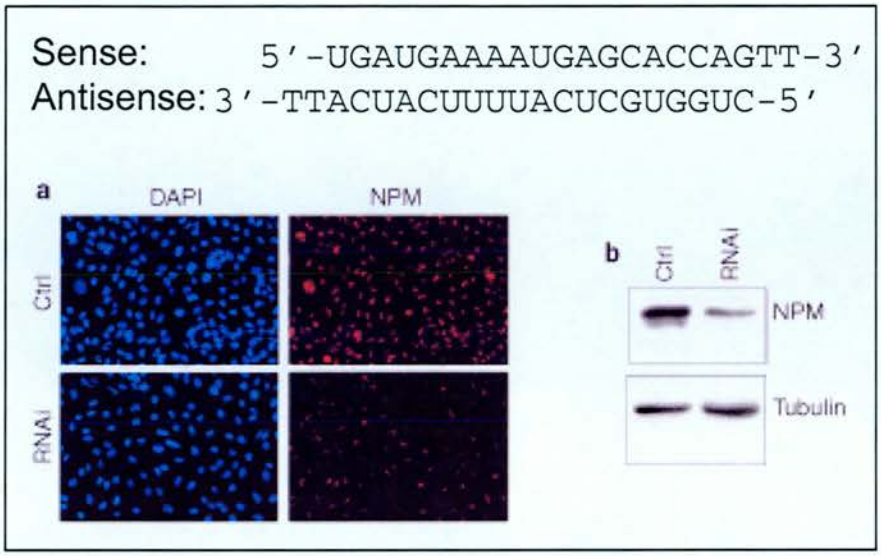


Figure 6.5: Analysis of Bax and nucleophosmin translocation during apoptosis by confocal microscopy. Bax protein (green) was diffusely localised within the cytosol in vehicle-treated (Control) cells, with staining becoming increasingly punctate during exposure to STS (2h) and displaying a predominantly mitochondria-associated staining pattern by 4h exposure to STS. In comparison, nucleophosmin (NPM; red) was nucleolar in control cells, but underwent re-localisation into the cytosol by 2h exposure to STS and remained cytosolic by 4h.

a.



b.

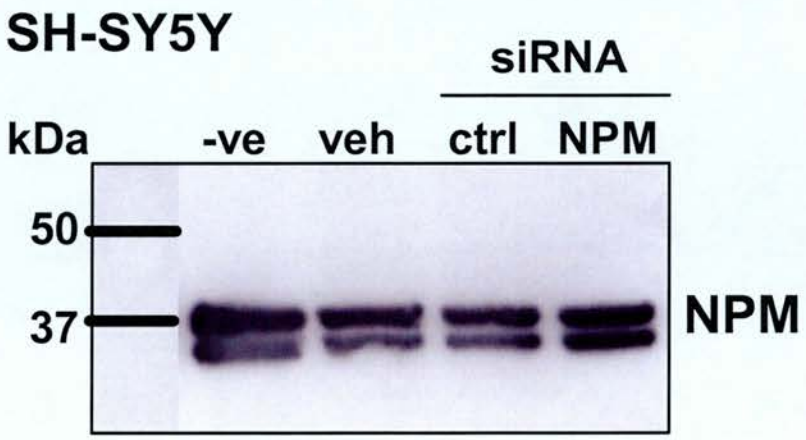


Figure 6.6. Failure to silence nucleophosmin protein expression in SH-SY5Y cells using a published siRNA oligonucleotide. Nucleophosmin (NPM) protein levels had been reportedly silenced by 60-70% using the siRNA sequence provided in **(a)**. This siRNA duplex was transfected into SH-SY5Y cells and nucleophosmin protein level measured at 48h by Western blot **(b)**. In this cell line, the siRNA sequence was ineffective at silencing nucleophosmin protein expression. Untreated cells (-ve); transfection reagent vehicle (veh); non-homologous siRNA (Ctrl); nucleophosmin-targeting siRNA (NPM). Image in (a) taken from Colombo *et al.*, 2002. Data are representative of at least three independent experiments.

out of five experiments performed in their laboratory using this siRNA sequence attained at least a 50% reduction in nucleophosmin protein expression. Therefore an alternative strategy utilising short hairpin siRNAs (shRNA) was used.

6.4.4. Attenuation of nucleophosmin protein expression using shRNA expression vectors

Four shRNA sequences were designed from the nucleophosmin cDNA ORF and ligated into the RNA polymerase III-driven expression vectors pSilencer-H1-Hygro and pSilencer-U6-Hygro – for sequence information see the Materials & Methods section 6.3.3 and Table 6.3. Correct insertion of the shRNA expression cassettes into the plasmid DNA vectors was confirmed by restriction digestion – see Figure 6.7.

Transfection of plasmid DNA into SH-SY5Y cells was optimised using either a ballistics approach (genegun) or a cationic lipid-based transfection reagent (Lipofectamine 2000) Transfection efficiency was determined using plasmid DNA expressing the YFP fluorophore and visualising successfully transfected fluorescent cells by confocal microscopy. A maximal transfection efficiency of approximately 30% was achieved using a plasmid DNA(μg) to Lipofectamine 2000 reagent (μl) ratio of 1:2 – see Figure 6.8.

During initial knockdown experiments, each shRNA vector was transfected separately into SH-SY5Y cells and their effect on nucleophosmin protein expression monitored by Western blot after 48h. From this, it became apparent that certain target sequences were more potent silencers of nucleophosmin expression than others, with silencing effects ranging from negligible to approximately 70% reduction in endogenous protein levels – see Figure 6.9a. The potency of individual siRNAs is known to be highly dependent on their target sequence within the mRNA strand (Reynolds *et al.*, 2004). On this basis, the use of several siRNA sequences targeting different regions within the same mRNA strand is recommended (Nature Cell biology editorial (2003)). However, when the four

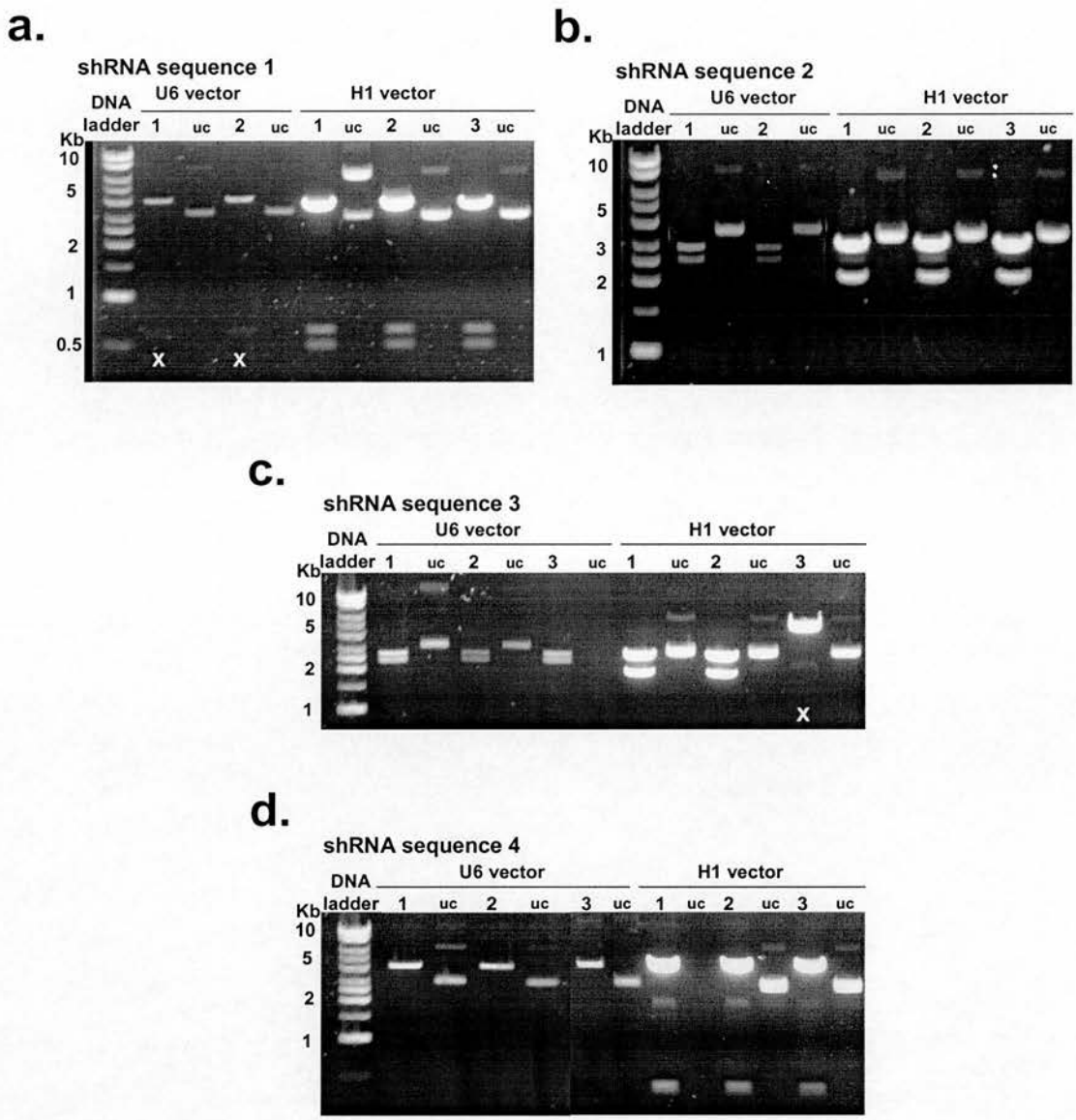


Figure 6.7. Validation of shRNA expression vectors by restriction digest. U6 and H1 promoter-driven expression vectors containing ligated shRNA sequences 1-4 were digested using insert-specific restriction enzymes to generate the following predicted DNA fragments (in bp): **Sequence 1 (a)** digested with BsmBI. U6 vector: 394 604 & 3855. H1vector: 490, 604 & 3430. **Sequence 2 (b)** digested with XmnI. U6 vector: 2306 & 2589. H1 vector: 1879 & 2687. **Sequence 3 (c)** digested with XmnI. U6 vector: 2307 & 2585. H1vector: 1880 & 2687. **Sequence 4 (d)** digested with Eco0109I. U6 vector: 296, 427 & 4170. H1vector: 523 & 4043. All ligations were successful, apart from those indicated by X's. Undigested plasmid DNA (uc) was run alongside each restriction digest.

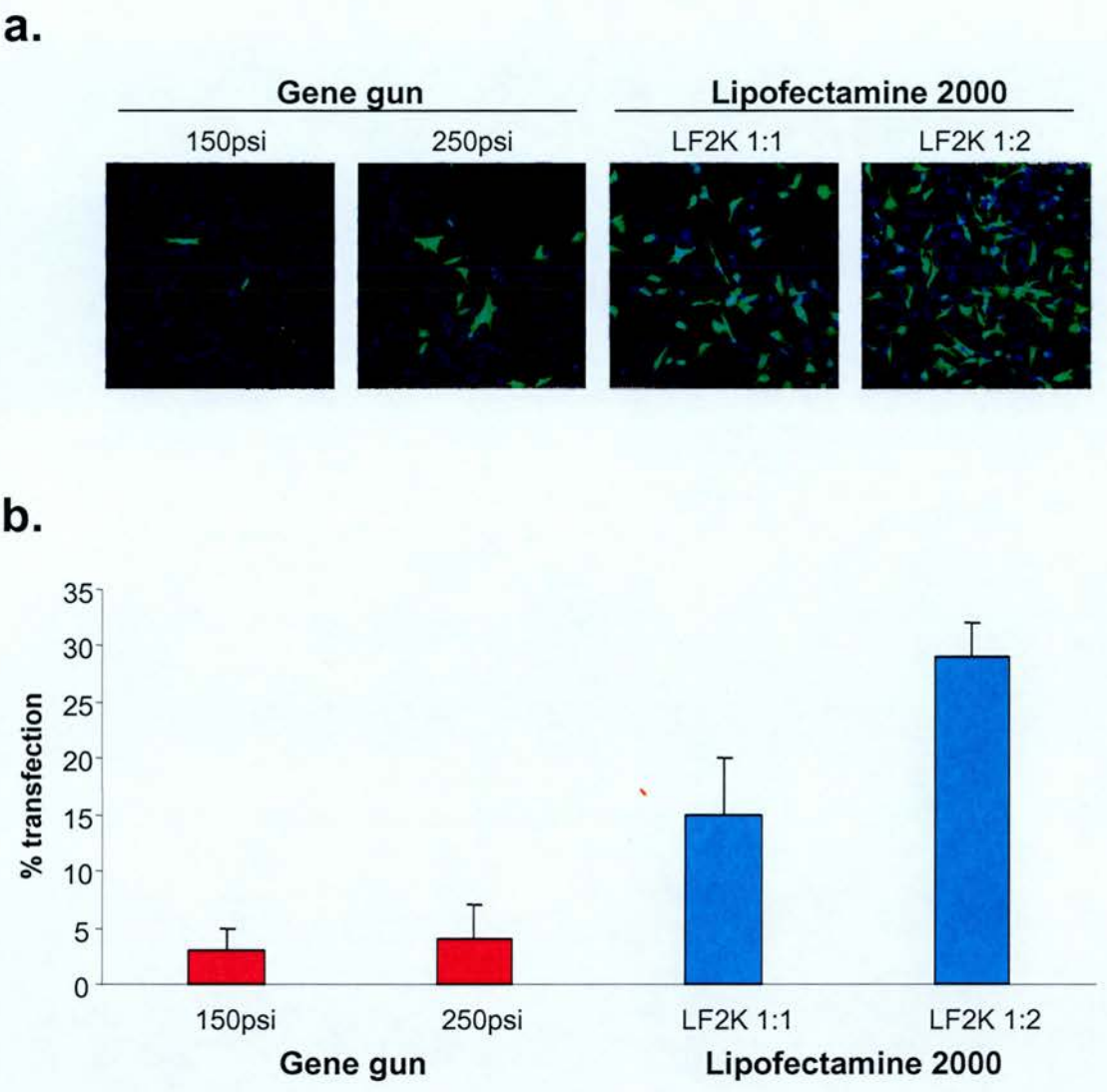


Figure 6.8. Optimising transfection of plasmid DNA into SH-SY5Y cells. YFP-expressing plasmid DNA was transfected into cultured SH-SY5Y cells using a genegun, pressurised to 150 or 250psi, or the cationic lipid-based transfection reagent, Lipofectamine 2000 (LF2K), at DNA (μg) to reagent (μl) ratios of 1:1 or 1:2. The percentage transfection efficiency was determined by counting the number of fluorescing cells within a defined field area using confocal microscopy **(a)**. Lipofectamine 200 reagent was more effective at transfecting plasmid DNA into SH-SY5Y cells, with the 1:2 ratio proving a maximal transfection efficiency of approximately 30% **(b)**. Data in (b) represents mean + s.e.m. of three independent experiments.

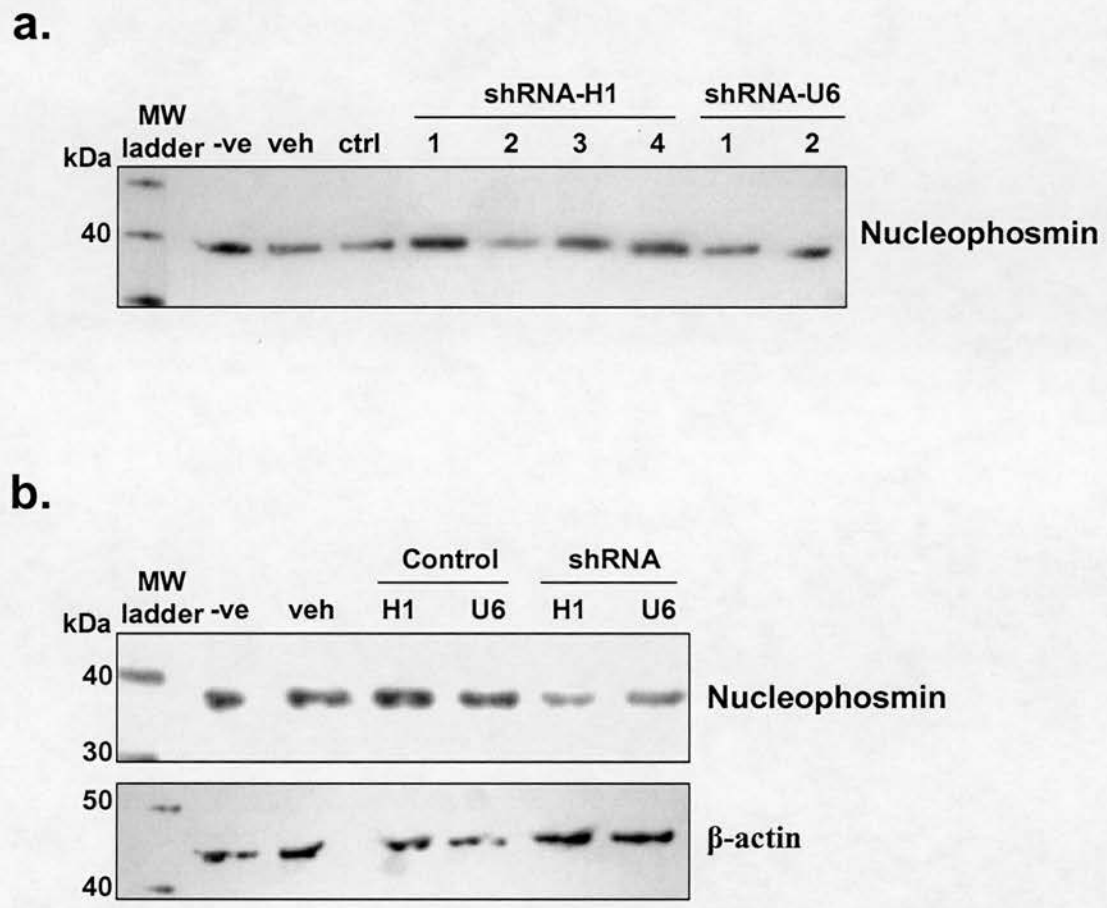


Figure 6.9. Assessment of shRNA-mediated knockdown of nucleophosmin protein expression by Western blot. shRNA expression vectors were transfected individually (shRNA-H1 sequences 1-4 and shRNA-U6 sequences 1 and 2) or combined into shRNA-H1 (H1) and shRNA-U6 (U6) pools. Nucleophosmin protein expression was assessed by Western blot at 72h post-transfection. Individually transfected shRNAs were variable in their silencing effect **(a)**. Pooled H1 shRNAs were the most effective at silencing nucleophosmin expression **(b)**. A control immunoblot for β-actin was included in the pooled shRNA experiment to demonstrate specificity of the silencing effect. –ve = untreated cells; veh = transfection reagent alone; ctrl = non-homologous siRNA control.

shRNA expression plasmids were combined together to form a pool, the silencing effect was found to be as high as the most potent shRNA vector alone, but more consistent in its effect – see Figure 6.9b. This approach also indicated that shRNAs expressed from the H1 promoter were more effective at silencing endogenous nucleophosmin protein expression than shRNAs expressed from the U6 promoter – see Figure 6.9b. Therefore, the H1 pool was used to silence nucleophosmin in all subsequent experiments using the shRNA approach.

Although shRNA-expressing vectors were capable of effectively reducing nucleophosmin protein expression, inconsistency of this effect proved problematic. This was possibly due to the different amounts of active siRNA generated within the transfected cell population between experiments. Thus, as commercially available siRNA products with improved design and efficacy became available, siRNA-based solutions for silencing nucleophosmin expression were evaluated again.

6.4.5. Optimisation of Dharmacon siRNA-mediated knockdown of nucleophosmin protein expression in SH-SY5Y cells

The siGenome smartPOOL system (Dharmacon) also utilises pools of several siRNAs each hybridising with a different region within the target mRNA strand. In order to maximise their silencing effect, two optimisation steps were performed: 1) to determine the optimal siRNA concentration delivered to the cell, and 2) to determine the optimal time-point at which to measure the silencing effect on protein expression.

To determine the lowest optimal concentration of siRNA that would silence nucleophosmin expression by at least 50% in SH-SY5Y cells compared to control-transfected cells, siRNA concentrations were titrated from 30nM up to 300nM. When 30nM of siRNA was transfected into SH-SY5Y cells, no silencing of nucleophosmin expression could be detected by Western blot – see Figure 6.10a. When 100nM siRNA was used, nucleophosmin protein expression was reduced to 44% in comparison to

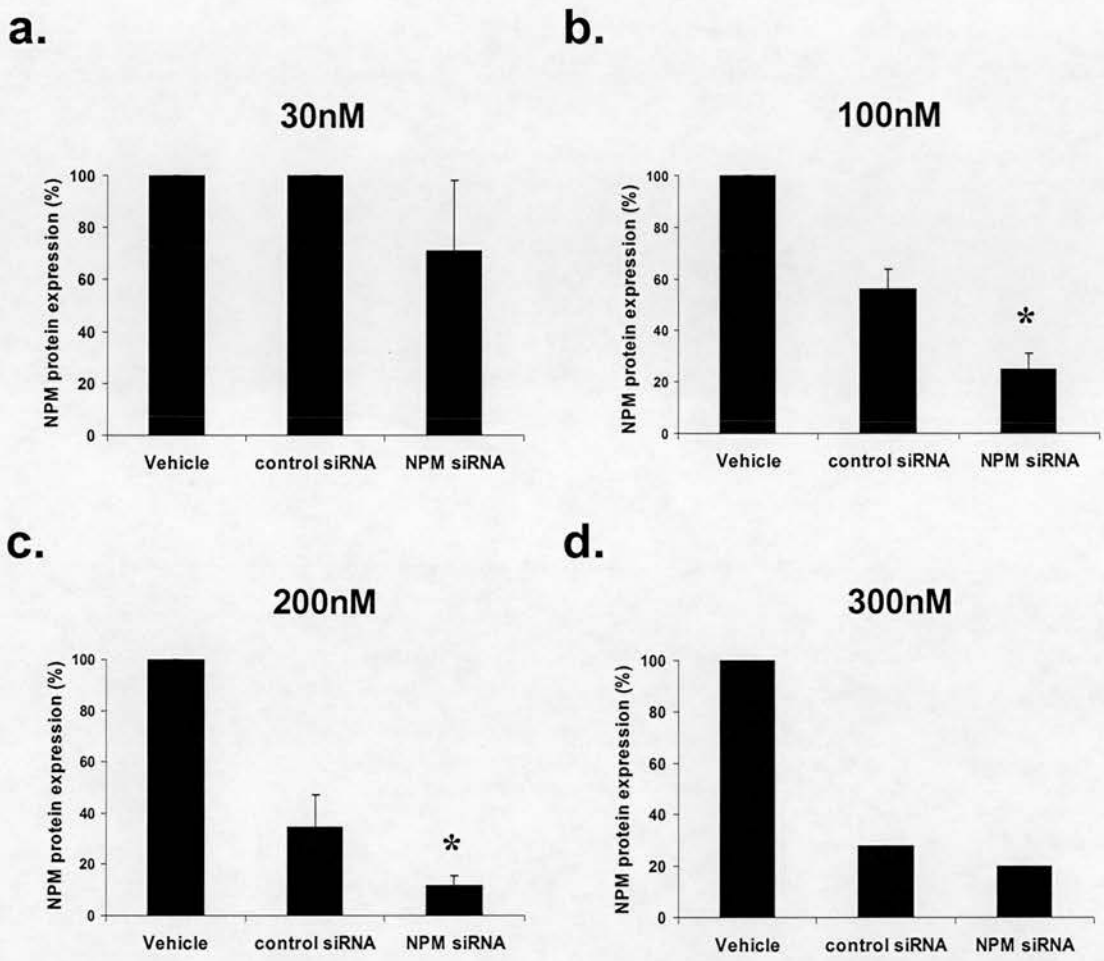


Figure 6.10. Optimising nucleophosmin siRNA concentration. 30nM **(a)**, 100nM **(b)**, 200nM **(c)** and 300nM **(d)** siRNA was transfected into SH-SY5Y cells and nucleophosmin (NPM) protein expression assessed by quantitative Western blot at 72 post-transfection. 100nM was the lowest siRNA concentration tested that achieved a greater than 50% reduction in protein expression compared to control siRNA-treated wells. Vehicle = transfection reagent alone. Data represent mean + s.e.m of three independent experiments, apart from the 300nM experiment (n=1). Data were analysed by one-way ANOVA with *post hoc* Dunnett's test. * = $p<0.05$ versus control siRNA.

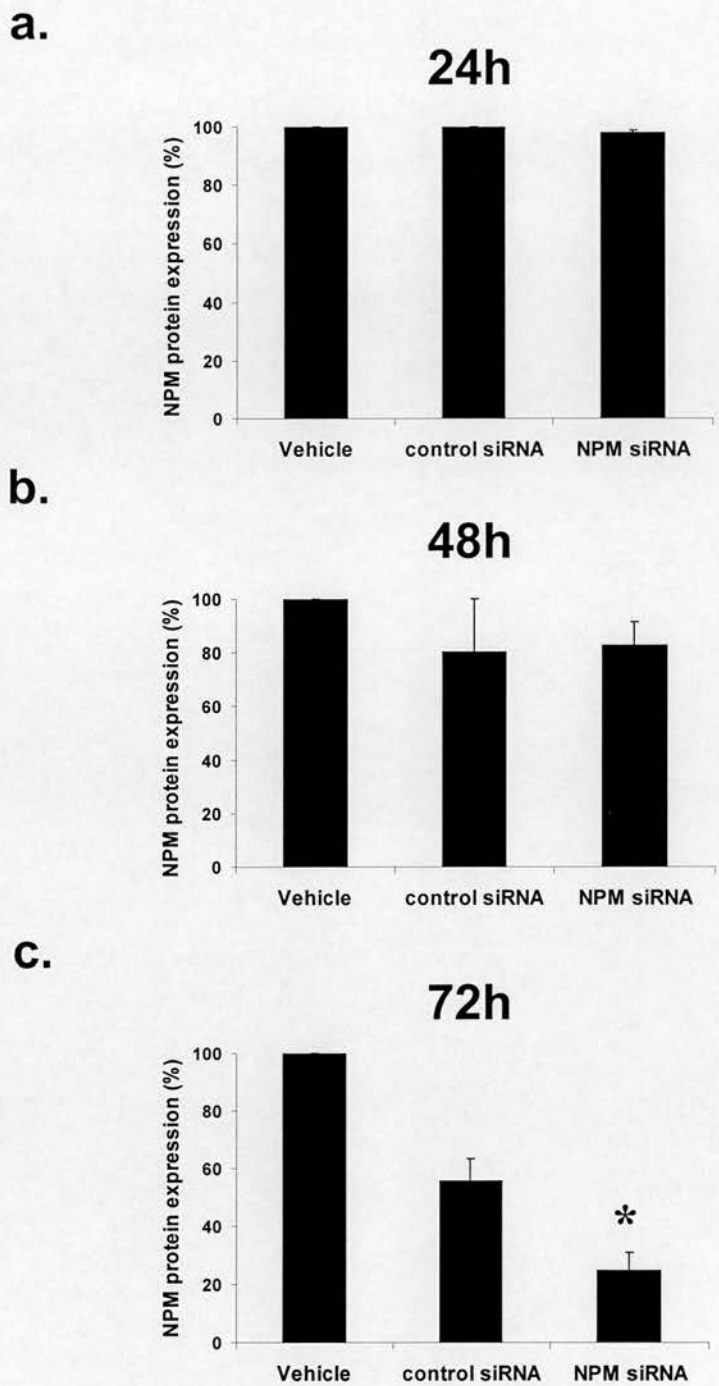


Figure 6.11. Optimising nucleophosmin siRNA assay time point. 100nM of siRNA was transfected into SH-SY5Y cells and nucleophosmin (NPM) protein expression assessed at 24h **(a)**, 48h **(b)** and 72h **(c)** by quantitative Western blot. From these studies, 72h was chosen as the optimal timepoint for assessing siRNA-mediated silencing of nucleophosmin protein expression. Vehicle = transfection reagent alone. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way ANOVA with *post hoc* Dunnett's test. * = $p<0.05$ versus control siRNA.

control siRNA-treated wells ($p<0.05$). Although nucleophosmin expression levels were markedly reduced compared to vehicle-treated cells when 200nM and 300nM siRNA was applied to cells, a significant reduction in expression compared to control siRNA-treated cells was not observed – see Figures 6.10b, c & d. High concentrations (in excess of 100nM) of siRNA have been shown to increase the risk of incurring non-specific "off-target" silencing of protein expression that can lead to errors in the interpretation of resulting phenotypic changes (Semizarov *et al.*, 2003). The inclusion of parallel experiments using non-homologous siRNA sequences (control siRNA) provides an indication of this non-specific ablation of protein expression (Elbashir *et al.*, 2001). Based on these data, a siRNA concentration of 100nM was chosen as the optimal concentration for all further nucleophosmin siRNA knockdown experiments.

The next optimisation step was to determine the optimal post-transfection timepoint to measure the knock-down effect. Typically, in an *in vitro*-based siRNA experiment, the impact on expression of the target protein is monitored between 24h and 72h post-transfection. This temporal window is thought to allow the introduced siRNA sufficient time to exert a measurable impact on protein expression whilst ensuring that the silencing effect is not diluted amongst an increasing daughter cell population as normal cell division runs its course (Ambion siRNA seminar, Edinburgh, November 2004). Therefore, SH-SY5Y cell lysate was collected for analysis by Western blotting at 24h, 48h and 72h following transfection of 100nM siRNA – see Figure 6.11. These experiments demonstrated that the longer the siRNA was allowed to mediate its effect, the more evident its impact on protein expression became. By 72h post-transfection, nucleophosmin protein expression in cells treated with 100nM siRNA had been reduced to 44% compared to control siRNA-treated wells ($p<0.05$). 72h post-transfection was therefore chosen as the timepoint at which to measure the biochemical consequences of reducing nucleophosmin protein expression within the intrinsic apoptotic pathway.

6.4.6. Reducing nucleophosmin expression by RNAi impairs the progression of the intrinsic apoptotic pathway but does not protect cells against STS-induced apoptosis

Endogenous nucleophosmin protein expression in SH-SY5Y cells was attenuated to approximately 50% using shRNA or siRNA-based RNAi approaches, compared to cells treated with control shRNAs or siRNAs, as described above. The biological consequences of this reduction on STS-induced apoptosis were then investigated by measuring Bax translocation, cytosolic cytochrome c levels, cleavage of pro-caspase-3, DNA fragmentation and cellular viability.

A comparative analysis of Bax translocation in control and nucleophosmin siRNA-treated SH-SY5Y cells during exposure to STS was attempted using fluorescent Bax antibodies and confocal microscopy. However, this approach was unsuccessful due to technical difficulties, most notably the incompatible seeding densities required for confocal analysis (optimally about 20% confluency at time of plating) and efficient transfection of siRNA into SH-SY5Y cells, which requires at least 60% confluency. As a result, the efficacy of the nucleophosmin siRNAs at reducing nucleophosmin protein expression in these experiments was less than that achieved in other experiments, hence the influence this had on Bax translocation could not be accurately determined.

However, the quantification of cytosolic cytochrome c levels and cleavage of procaspase-3 in cells treated with nucleophosmin-targeting siRNAs was achievable. These experiments demonstrated that reducing nucleophosmin protein levels to 51% as a result of RNAi attenuated cytosolic cytochrome c levels to 41% and procaspase-3 cleavage to 54% (both $p<0.05$) in comparison to control siRNA-treated cells - see Figure 6.12. However, analysis of DNA fragmentation and cellular viability in nucleophosmin siRNA-treated cells showed little or no overall corresponding protective effect when compared to control cells – see Figure 6.13.

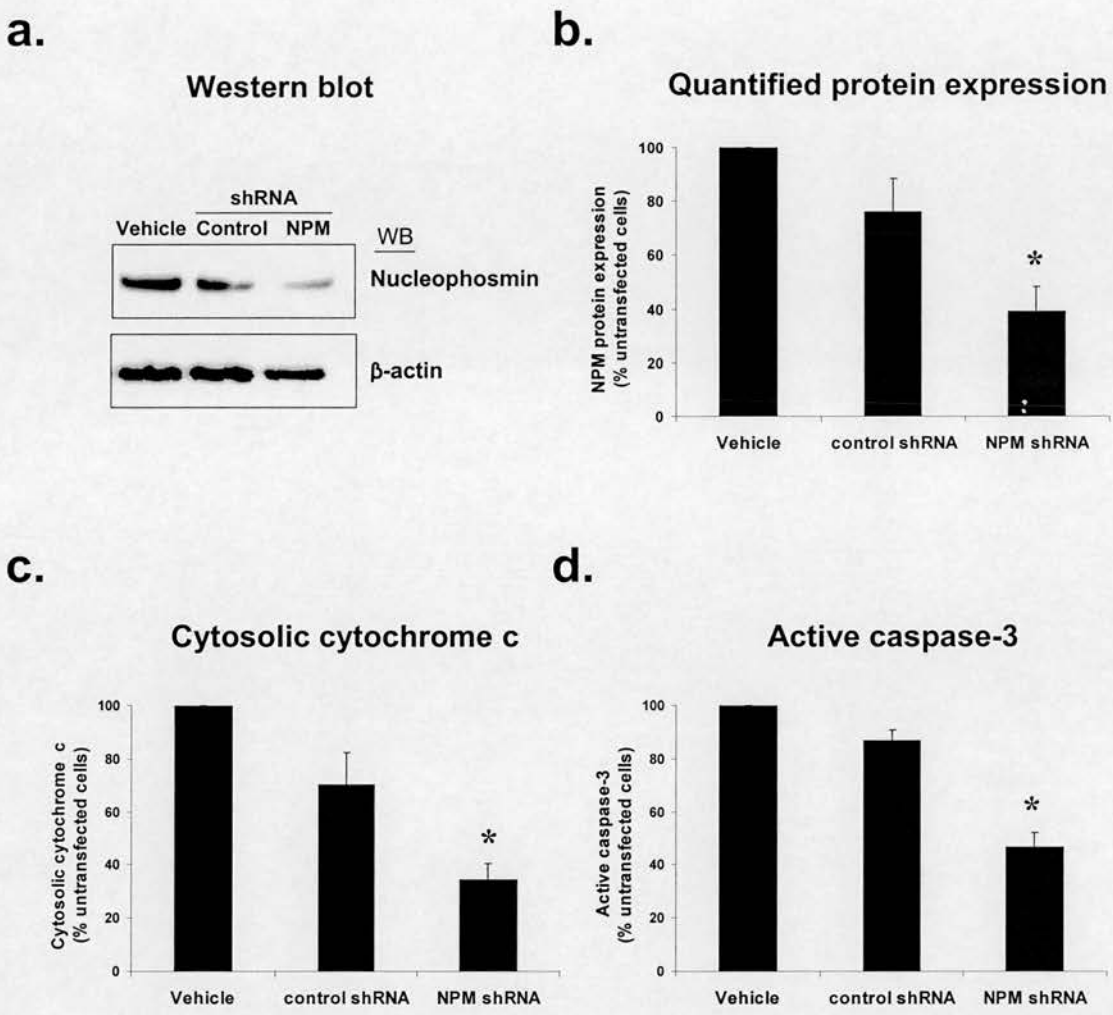


Figure 6.12. Effect of RNAi-mediated knock down of nucleophosmin expression on STS-induced apoptosis. Nucleophosmin (NPM) protein expression in SH-SY5Y cells was silenced by approximately 50% compared to control siRNA-treated cells as assessed by quantitative Western blot **(a & b)**. This resulted in a 50% reduction in cytochrome c released from the mitochondria **(c)** and a 50% reduction in activate caspase-3 **(d)** in these cells. Vehicle = transfection reagent alone. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way ANOVA with *post hoc* Dunnett's test. * = $p<0.05$ versus control shRNA.

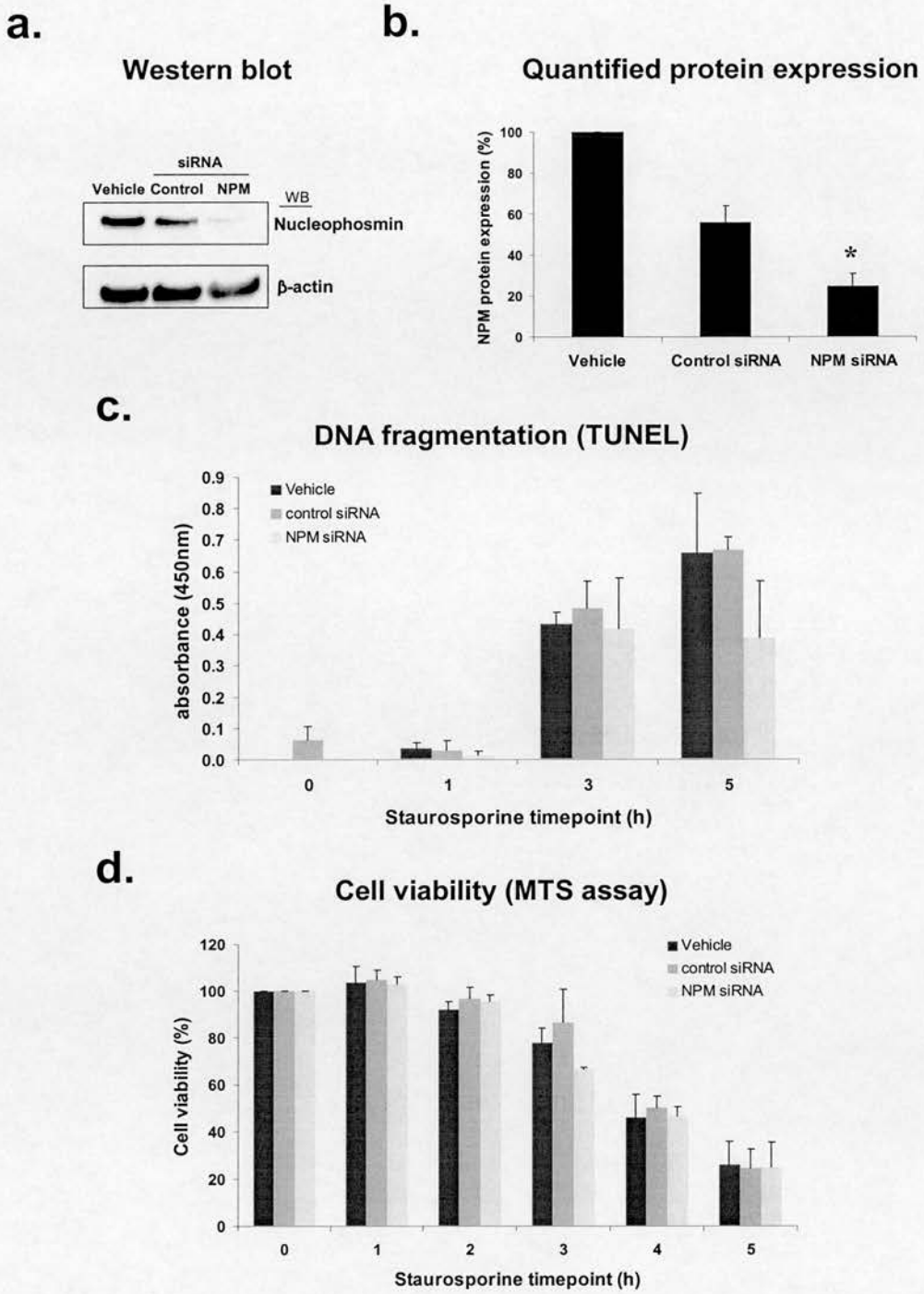


Figure 6.13. Assessing protective effect of nucleophosmin siRNA against STS-induced apoptosis. Nucleophosmin (NPM) protein levels were reduced by 50% compared to control cell using siRNA as assessed by quantitative Western blot **(a & b)**. Internucleosomal DNA fragmentation was measured over a 5h timecourse by TUNEL ELISA **(b)** and metabolic cell death measured by MTS assay **(c)**. No protective effect was observed in nucleophosmin siRNA-treated cells when compared to control siRNA-treated cells. Vehicle = transfection reagent alone. Data represent mean + s.e.m of three independent experiments. Data were analysed by one-way and two-way ANOVA with *post hoc* Dunnett's test. * = $p<0.05$ versus control siRNA.

6.5 Discussion

In chapter 4, an interaction between nucleophosmin and the pro-apoptotic protein Bax was identified within the human neuroblastoma SH-SY5Y cell line. In Chapter 5, the interaction between Bax and nucleophosmin was investigated at the molecular level using recombinant proteins. In this chapter, the biological relevance of the Bax-nucleophosmin interaction was investigated using RNA interference (RNAi) in conjunction with biochemical analyses of the intrinsic apoptotic pathway using the *in vitro* cell model established in Chapter 3.

As a molecular chaperone and transport factor, nucleophosmin has been proposed to direct the intracellular trafficking of several proteins bearing a nuclear localisation signal, such as pRb (Takemura *et al.*, 2002) and the HIV Tat protein (Li, 1997). Although these chaperoning activities of nucleophosmin take place within the confines of the nucleus, nucleophosmin has also been observed to constantly shuttle between the nucleus and the cytoplasm (Borer *et al.*, 1989) and, from a more recent observation, to undertake this translocation during aloe-emodin-induced apoptosis in a lung carcinoma cell line (Lee *et al.*, 2005). Furthermore, from studies performed within the ACE laboratory, nucleophosmin has been demonstrated to undergo re-distribution *in vivo* from the nucleus into the cytoplasm in the mouse cortex following transient focal cerebral ischemia (Kerr *et al.*, 2006). Significantly, this translocation event was observed to occur prior to the translocation of Bax to the mitochondrial surface. Thus, although Bax and nucleophosmin occupy distinct cellular compartments under normal conditions (Bax in the cytosol and nucleophosmin in the nucleolus), the documented re-distribution of nucleophosmin into the cytosol during cellular stress provides an opportunity for these two proteins to interact. In this chapter, the analysis of Bax and nucleophosmin re-distribution during STS-induced apoptosis in SH-SY5Y cells by confocal microscopy provides additional evidence to suggest that a functional interaction between Bax and nucleophosmin occurs during apoptosis. From these

studies, Bax and nucleophosmin were observed to occupy the cytosolic compartment within a proportion of cells by 2h exposure to STS, whereupon Bax underwent translocation to mitochondria as evidenced by the emergence of a mitochondria-associated punctate staining pattern by 4h. However, this observation, although consistent with the hypothesis that nucleophosmin contributes to the Bax translocation event, does not necessarily imply that the translocation of Bax is a direct result of the appearance of nucleophosmin in the cytosol.

Therefore, having established that Bax and nucleophosmin could interact during STS-induced apoptosis, attention turned to determining the functional consequences of such an interaction in the context of the intrinsic apoptotic pathway, in which Bax plays a central and rate-determining role (Wei *et al.*, 2001; Zong *et al.*, 2001; Cheng *et al.*, 2001). Knockdown of nucleophosmin protein expression using RNAi attenuated progression of the intrinsic apoptotic pathway in SH-SY5Y cells, as seen by a concomitant reduction in mitochondrial cytochrome c release and cleavage of the apical caspase zymogen, procaspase-3. Together, these data suggest that nucleophosmin is required for Bax to exert its physiological role within the intrinsic apoptotic pathway upstream of mitochondrial membrane depolarisation.

Similar roles have recently been attributed to several other Bax-binding proteins, all of which have been discovered since this thesis was begun. Such proteins include the apoptosis-associated speck-like protein (ASC) (Ohtsuka *et al.*, 2004), Bax-interacting factor-1 (Bif-1) (Takahashi *et al.*, 2005) and the BH3 only-like protein, modulator of apoptosis-1 (MAP-1) (Baksh *et al.*, 2005). Investigating the ability of these proteins to promote Bax-mediate apoptosis in response to a variety of apoptotic insults indicates that their influence is highly dependent on the type of apoptotic inducer or apoptotic pathway stimulated. For example, knockdown of ASC was effective at limiting apoptosis induced by p53 or etoposide (Ohtsuka *et al.*, 2004), and knockdown of Bif-1 expression was protective against various intrinsic death stimuli (Takahashi *et al.*, 2005). In contrast, however, inhibiting MAP-1 conferred protection against the cell death

receptor pathway-activating ligand, TNFα, but not to the mitochondrial pathway inducers STS or etoposide (Baksh *et al.*, 2005).

This disparate picture in the efficacy of Bax-binding proteins may help to explain why, in our hands, knockdown of nucleophosmin attenuated progression of the intrinsic apoptotic pathway in SH-SY5Y cells, whereas in response to other intrinsic pathway-inducing stimuli, such as hypoxia or NGF deprivation, nucleophosmin knockdown has instead been found to precipitate apoptosis (Li *et al.*, 2004b; Ahn *et al.*, 2005).

A similarly contradictory picture emerges when the proposed roles of nucleophosmin in regulating the activities of other key apoptogenic factors are examined. Nucleophosmin has recently been implicated as a key determinant in the response of the tumour suppressor proteins, $p19^{ARF}$ and p53, to genotoxic stress (Itahana *et al.*, 2003; Kurki *et al.*, 2004; Bertwistle *et al.*, 2004; Kuo *et al.*, 2004; Korgaonkar *et al.*, 2005) What role nucleophosmin plays in regulating $p19^{ARF}$ function appears to be highly dependent on nucleophosmin protein expression. For instance, when nucleophosmin is aberrantly over-expressed, as it is in many cancers, it functions as an oncogene by inhibiting $p19^{ARF}$'s stimulatory effect on p53 through sequestration of $p19^{ARF}$ in the nucleolus (Korgaonkar *et al.*, 2005). Conversely, in non-cancerous cells, nucleophosmin exerts a tumour-suppressive role (Lambert and Buckle, 2006; Grisendi *et al.*, 2006), partly through binding to and maintaining the $p19^{ARF}$ protein in a stable form (Kuo *et al.*, 2004; Colombo *et al.*, 2005). As for p53, numerous independent reports have demonstrated that it directly interacts with nucleophosmin (Colombo *et al.*, 2002; Maiguel *et al.*, 2004; Li *et al.*, 2005; Lambert and Buckle, 2006). However, what appears to be less certain is the functional consequence of this interaction, with some groups implicating nucleophosmin as a crucial stabiliser of p53 activity during DNA damage-induced apoptosis (Colombo *et al.*, 2002; Kurki *et al.*, 2004) and others insisting that its upregulation inhibits the p53 response (Maiguel *et al.*, 2004; Li *et al.*, 2005). Again, the type of apoptotic stimulus, together with its intensity and duration of effect, appears to be highly influential in dictating whether the contribution made by nucleophosmin to

p53 function is favourable or detrimental to cell survival. This ambiguity could not be resolved from examining the phenotype resulting from *Npm1*$^{-/-}$ mice, which have recently been developed independently by two research groups (Colombo *et al.*, 2005; Grisendi *et al.*, 2005b). In both studies, disruption of nucleophosmin expression led to embryonic lethality at mid-gestation, in testament to its crucial role in haematopoiesis and in the maintenance of genomic stability. Analysis of these embryos revealed the presence of extensive apoptotic cell death together with stabilisation of p53. However, although this finding may support a role for nucleophosmin in suppressing p53 activity, it is more likely that p53 in this case is responding to the extensive DNA damage and aberrant mitotic spindle formation arising from nucleophosmin's absence in these embryos (Grisendi *et al.*, 2006).

Despite reducing cytochrome c release and procspase-3 cleavage, siRNA-mediated knockdown of nucleophosmin did not protect cells against STS-induced apoptosis, as evidenced by the observed extent of DNA fragmentation and decrease in cellular viability. Two possible explanations may account for these findings: 1) incomplete silencing of nucleophosmin protein expression, and 2) activation of Bax-independent apoptotic pathways. The RNAi methodologies employed against nucleophosmin did not silence nucleophosmin expression, but instead reduced expression to approximately half normal levels. Consequently, the intrinsic apoptotic pathway was still able to proceed, albeit at a diminished rate, towards executing apoptosis. The apoptotic inducer used in these experiments, STS, although widely used to stimulate activation of the intrinsic apoptotic pathway in a variety of cell types (Desagher *et al.*, 1999; McGinnis *et al.*, 1999; Eskes *et al.*, 2000) (Krohn *et al.*, 1999; Poppe *et al.*, 2001; Ahlemeyer *et al.*, 2002),(Tafani *et al.*, 2001; Yuste *et al.*, 2002) is nevertheless a broad-spectrum kinase inhibitor, and may therefore trigger parallel, Bax-independent, pathways leading to apoptotic cell death. One further explanation is that other pro-apoptotic Bcl-2 family proteins, such as Bak, can permeabilise the OMM in the absence of Bax, as has previously been proposed (Lindsten *et al.*, 2000; Wei *et al.*, 2001). However, the relative contribution made by Bax and Bak to the intrinsic pathway is thought to be

dependent on cell type (Arnoult *et al.*, 2004). For example, Bax has been shown to be required for apoptosis initiated by a variety of intrinsic stress stimuli in cultured human epithelial cancer cells (Theodorakis *et al.*, 2002; Mikhailov *et al.*, 2003) and it has been demonstrated that Bax is essential for several neuronal cell populations to initiate the intrinsic apoptotic pathway during development (Deckwerth *et al.*, 1996; White *et al.*, 1998). Furthermore, the attenuation in cytosolic cytochrome c and procaspase-3 cleavage observed in this thesis as a direct consequence of ablating nucleophosmin protein expression with siRNA is consistent with a Bax-dependent route to OMM permeabilisation in this experimental model.

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Overall, the data presented within this chapter demonstrate that a targeted reduction in expression of the novel Bax-binding protein, nucleophosmin, although unable to rescue neuronal cells from STS-induced apoptosis, is sufficient to attenuate key biochemical events associated with the activation and translocation of Bax to the mitochondrial surface.

6.6 Summary

In this chapter, the biological significance of the Bax-nucleophosmin interaction was investigated using a neuronal cell model of the intrinsic apoptotic pathway. An interaction between endogenous Bax and nucleophosmin was confirmed by reciprocal co-immunoprecipitation, and evidence that nucleophosmin translocates from the nucleolus into the cytosol during STS-induced apoptosis was provided by confocal microscopy. The development of an RNAi-based strategy to elucidate the functional significance of the Bax-nucleophosmin interaction within the intrinsic apoptotic pathway was then implimented. Expression of endogenous nucleophosmin protein in SH-SY5Y cells was reduced to approximately 50% of that found in the appropriate control-treated cells using RNAi. How nucleophosmin-deficient cells responded to STS-induced apoptosis was then investigated using the *in vitro* cell-based model of the intrinsic apoptotic pathway established in chapter 3. As a result of reducing nucleophosmin

protein levels by RNAi, STS-induced cytochrome c release and procaspase-3 cleavage were attenuated, supporting the hypothesis that nucleophosmin is required for the apoptogenic activity of Bax, possibly during the translocation process. However, due to experimental limitations, siRNA-mediated knockdown of nucleophosmin protein expression did not confer protection against STS-induced apoptotic cell death. Overall, the identification of nucleophosmin as a novel Bax binding protein and potential regulator of stress-induced neuronal apoptosis represents an important new insight into the molecular mechanisms underlying the unwarranted apoptotic cell death associated with neurodegenerative diseases and stroke pathology.

CHAPTER 7: Summary & Conclusions

7.1. Summary & Conclusions

Bax, a pivotal pro-apoptotic member of the Bcl-2 protein family, is an essential requirement for apoptosis within the developing mammalian nervous system (Deckwerth *et al.*, 1996; White *et al.*, 1998) and has been implicated in contributing to the pathological apoptotic cell death associated with neurodegenerative disorders (Isenmann *et al.*, 1997; Zhang *et al.*, 1998; Tuunanen *et al.*, 1999; Mattson, 2000). The molecular events dictating the translocation of Bax to the mitochondrial surface remain poorly understood, but the reliance of Bax on the enforced exposure of a hydrophobic membrane-targeting domain (Wolter *et al.*, 1997; Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000; Schinzel *et al.*, 2004b) suggests that chaperone proteins may play a critical role in this event.

The apoptogenic function of Bax is utilised by the intrinsic pathway to apoptosis, which, in the central nervous system, is initiated by the withdrawal of neurotrophic factors during development (Putcha *et al.*, 2002) and by various cellular stress, including hypoxia, perturbed calcium homeostasis and the accumulation of toxic protein aggregates during the onset and progression of neurodegenerative disorders (Stefanis, 2005). In order to accurately study the apoptotic pathway associated with such disorders, an *in vitro* cell-based model of the intrinsic apoptotic pathway was established using the human neuroblastoma SH-SY5Y cell line and the intrinsic apoptotic pathway inducer, staurosporine, as described in Chapter 3.

The proteomics-based approach described in Chapter 4 aimed to identify novel Bax-binding proteins within whole cell lysates in which Bax protein expression levels represented those encountered under endogenous conditions. Furthermore, the use of detergents, with the exception of the zwitterionic detergent CHAPS, was avoided as this is known to cause artificial interactions as a consequence of inducing Bax

conformational changes (Hsu and Youle, 1998). Six proteins (Hsp60, Hsc71, GRP78, vimentin, tubulin & β-actin) were consistently identified as Bax co-precipitants from 2D gel and mass spectrometry analyses. Subsequent co-immunoprecipitation-based studies confirmed interactions between Bax and Hsp60, vimentin and β-actin.

The identification of Bax-binding proteins originating from the Hsp family and the cytoskeleton provides additional insight into how quiescent Bax is maintained inactive within the cytosol prior to receiving an apoptosis-inducing stimulus. Several Hsp family proteins are known to be important inhibitors of apoptosis, acting at various checkpoints within the intrinsic apoptotic pathway (Garrido *et al.*, 2001; Beere, 2004), and Hsp60 has been previously identified as a Bax-retention factor in human cardiac cells (Kirchhoff *et al.*, 2002). Furthermore, the novel association between Bax and the cytoskeletal proteins, vimentin and β-actin, could also provide a mechanistic insight into how Bax is able to sense and respond rapidly to cellular damage – perhaps utilising a similar mechanism to the other cytoskeleton-sequestered pro-apoptotic Bcl-2 family proteins, Bmf-1 and Bim (Puthalakath *et al.*, 1999; Puthalakath *et al.*, 2001).

In Chapter 4, Hsp60 and β-actin were also identified as proteins interacting with the Bax C-terminal trans-membrane domain which is thought to regulate the targeting of Bax to the OMM during apoptosis (Nechushtan *et al.*, 1999; Suzuki *et al.*, 2000). As a key molecular chaperone involved in protein structure maturation and refolding, Hsp60 could regulate the exposure of the Bax C-terminal tail and subsequently set a threshold for Bax activation in neuronal cells, as has been previously reported in cardiac cells (Kirchhoff *et al.*, 2002; Knowlton and Gupta, 2003). The identification of β-actin as a Bax C-terminal-binding protein, however, is a novel finding and adds additional support to the recently proposed role for the actin cytoskeleton in orchestrating the translocation of Bax, and perhaps other Bcl-2 family proteins, to the mitochondrial surface during the early phase of apoptosis (Tang *et al.*, 2006).

The key finding of this thesis, and the focus of Chapters 5 and 6, was the identification of the molecular chaperone, nucleophosmin, as a novel binding partner to the Bax C-terminal membrane-targeting domain within SH-SY5Y cells. Nucleophosmin was initially identified as a protein binding specifically to a S184V mutant Bax C-terminal using affinity chromatography, as described in Chapter 4. This preferential binding of nucleophosmin to the activated Bax conformation was later confirmed in Chapter 5 using recombinant proteins in a cell-free proximity assay where nucleophosmin was observed to bind only to detergent-activated Bax. Interaction between endogenous proteins during STS-induced apoptosis was also confirmed by reciprocal co-immunoprecipitation in Chapter 6. Furthermore, that this interaction was a biologically relevant interaction was demonstrated in Chapter 6 using confocal microscopy, to show that Bax and nucleophosmin co-localised within the cytosol of SH-SY5Y cells during STS-induced apoptosis, and RNA interference, which showed that nucleophosmin was required for Bax to release cytochrome c across the OMM and initiate downstream caspase activity. An overview of how the Bax-binding proteins identified in this thesis could regualate the bioactivity of Bax in SH-SY5Y cells is provided in Figure 7.1.

During the course of this thesis, the identification of several Bax-binding proteins out-with the Bcl-2 protein family has been reported in the literature (Gustafsson et al, 2004; Mao et al, 2004; Gotoh et al, 2004; Zhang et al, 2005; Gardai et al, 2004; Takahashi et al, 2005; Ohtsuka et al, 2004). The majority of these have been attributed with inhibitory roles, mainly through sequestration of Bax as an inactive protein within the cytosol. Furthermore, several of these regulatory proteins, such as ARC (Koseki *et al.*, 1998), αA-crystallin (Bloemendal, 1982), 14-3-3σ (Prasad *et al.*, 1992) and humanin (Hashimoto *et al.*, 2001; Tajima *et al.*, 2002) exhibit a restricted tissue distribution, suggesting that Bax may be regulated by different proteins according to the cell type. Some of the Bax-binding proteins identified within this thesis, therefore, could have a specific role in regulating Bax within neuronal cells. However, before this could be established, the ability of such proteins to bind Bax within cell lines originating form other tissue types would need to be investigated.

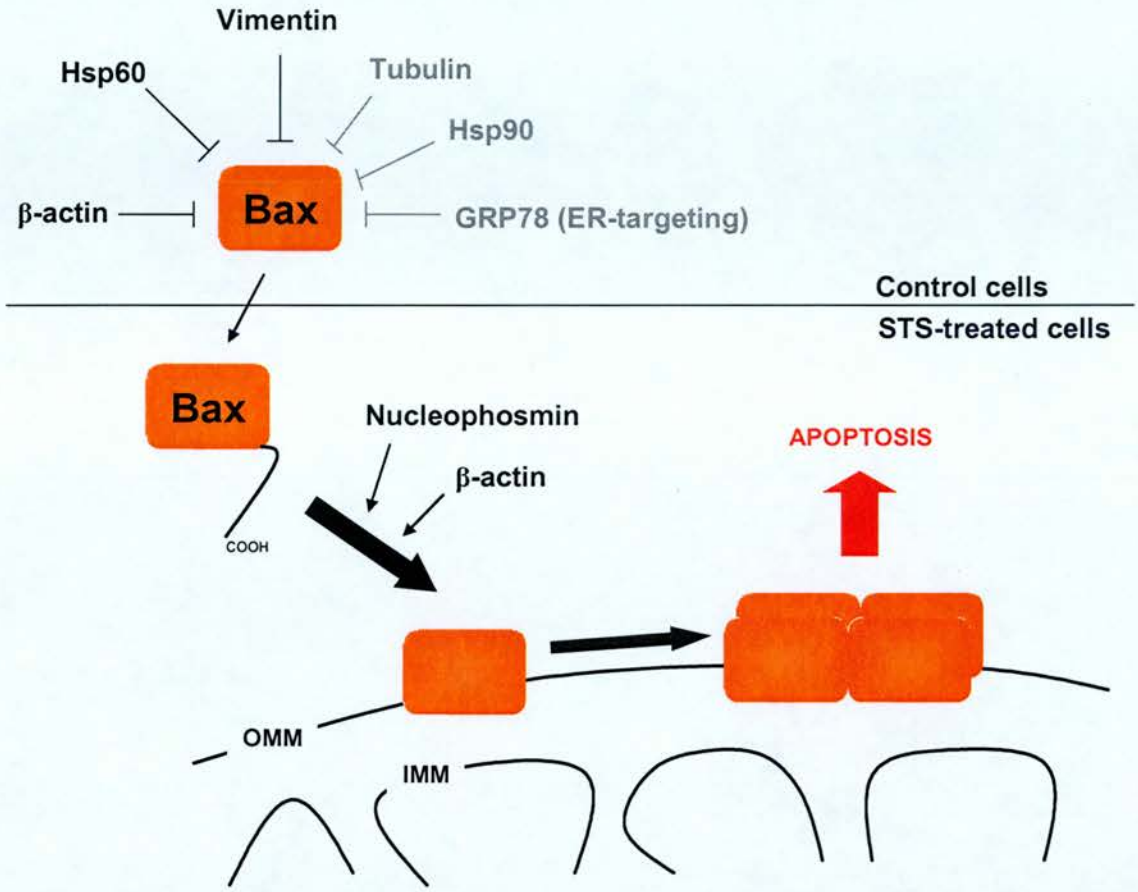

Fig. 7.1. Proposed model of Bax regulation during STS-induced apoptosis in SH-SY5Y cells. In the experimental cell model used in thesis, several heat shock and cytoskeletal proteins were identified from 2D gel electrophoresis as potential cytosolic Bax retentive factors in unstressed (control) cells. However, three of these proteins, Tubulin, Hsp90 and the ER chaperone, GRP78, cold not be confirmed as Bax-binding proteins. Affinity chromatography experiments identified several proteins specifically binding to the putative Bax C-terminal membrane-targeting domain, among them the molecular chaperone, nucleophosmin, and the cytoskeletal protein, β-actin. A direct interaction between nucleophosmin and the activated Bax conformation was confirmed using recombinant proteins in a proximity assay. Recently published data has identified a potential role for the actin cytoskeleton in the Bax translocation event (Tang *et al.*, 2006). Data from this thesis supports the hypothesis that nucleophosmin is a novel regulator of the apoptogenic function of Bax, and is required for Bax to elicit the mitochondrial release of apoptogenic factors in SH-SY5Y cells exposed to STS.

Significantly, as discussed in Chapter 6, three recently identified binding proteins, Bif-1, ASC and the BH3-like protein, MAP-1, have been suggested, as nucleophosmin has in this thesis, to promote the apoptogenic activity of Bax (Ohtsuka *et al.*, 2004; Takahashi *et al.*, 2005; Baksh *et al.*, 2005). Until the identification of such proteins, Bax was thought to rely solely on the functional interaction with other pro-apoptotic Bcl-2 family proteins, such asBid/tBid, Bim and PUMA (Wang *et al.*, 1996; Desagher *et al.*, 1999; Eskes *et al.*, 2000; Marani *et al.*, 2002; Cartron *et al.*, 2004). The identification of Bif-1, ASC, MAP-1 and, from this thesis, nucleophosmin, supports an emerging hypothesis that Bax relies on a functional collaboration with several different proteins, the involvement of each perhaps depending on cell type and apoptotic stimulus, in order to fulfil its apoptogenic role at the mitochondrial surface (Lucken-Ardjomande and Martinou, 2005).

The *in vitro*-derived data presented within this thesis has recently been corroborated *in vivo* using a mouse model of ischemic stroke (Kerr *et al.*, 2006). Histological assessment of mouse brains taken from animals subjected to 30mins transient occlusion of the middle cerebral artery (MCA) revealed the presence of activated Bax 3h following onset of the ischemic insult. Analysis of the subcellular distribution of both Bax and nucleophosmin within the cortex at this time point was then performed by isolating nuclear, mitochondrial and cytosolic fractions and measuring relative proteins levels in each by quantitative Western blot. Using this approach, while Bax localisation remained relatively unaltered between sham operated and the ischemic cortex, nucleophosmin was observed to have undergone translocation from the nuclear compartment into the mitochondrial and cytosolic fractions by 3h post-insult. This data is in agreement with the *in vitro* confocal microscopy-based analysis presented within Chapter 6 of this thesis and therefore represents a promising confirmation that the Bax-nucleophosmin interaction is of physiological importance *in vivo* and that it may therefore represent an important target for therapeutic intervention in diseases associated with deregulated apoptosis.

Overall, the key findings presented within this thesis suggest that: 1) Bax is sequestered within the cytosol of neuronal cells in an inactive state by association with the anti-apoptotic protein, Hsp60, and the cytoskeletal proteins, vimentin and β-actin, 2) Hsp60 and β-actin specifically interact with the Bax C-terminus and may function to either regulate its exposure or assist in the translocation of Bax to the OMM, 3) The molecular chaperone, nucleophosmin, engages in a novel interaction with the C-terminal domain of activated Bax, 4) Nucleophosmin is required for the apoptogenic activity of Bax upstream of mitochondrial membrane permeabilisation during STS-induced apoptosis in SH-SY5Y cells and, as such, represents a novel target for therapeutic intervention of intrinsic apoptotic neuronal cell death.

7.2. Future studies

During the course of this thesis, several further studies were considered but were not performed due to constraints placed on these studies by time and available resources. However, they are presented here as potential future studies that could be performed as part of a research project to add further insight into the role of Bax binding proteins, and particularly nucleophosmin, in regulating the apoptogenic activity of Bax.

1) Establishing the functional contribution made by Hsp60, vimentin and ß-actin to the bioactivity of Bax within SH-SY5Y cells.
2) Investigating the interaction between Bax and nucleophosmin, and the functional consequences of this interaction, using a variety of cell lines and apoptotic inducers.
3) Using subcellular fractionation techniques to determine the relative cellular translocation timecourses of Bax and nucleophosmin during STS-induced apoptosis
4) Using RNAi methodologies targetting Bax to determine the significance of Bax in STS-induced apoptosis in SH-SY5Y cells.

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